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STUDIES ON THE "DOUBLE NUCLEUS" IN THE EARLY DEVELOPMENTAL STAGE OF *BUFO VULGARIS*

I. CYTO-EMBRYOLOGICAL CONSIDERATIONS ON ITS POSSIBLE PARTICIPATION IN THE NORMAL COURSE OF CELL MULTIPLICATION

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I. INTRODUCTION

In studying the cell number and the quantitative process of cell multiplication in early developmental stages of *Bufo vulgaris*, the present author noticed in the gastrula frequent occurrence of two nuclei attached side by side in one cell body. In this series of papers the term "double nucleus" is used to designate these figures. Similar figures have been reported by several authors (CHILD, GLASER, GURWITSCH, STOUGH, etc.) from embryos of many forms and customarily called "amitosis." For instance, GODA (1929, p. 56) stated, in his extensive studies on the embryonic cells of *Bufo vulgaris*, that amitoses were often observed besides regular mitotic figures in the gastrula. Although opinions prevail that the nature of the amitosis in adult tissues is more or less pathological (GODLEWSKI, 1909; WILSON, 1925; GRESSON, 1949; etc.), its significance for the actual course of cell multiplication during the embryonic development is still quite obscure.

From a morphological viewpoint, it might hardly be supposed that the amitosis leads always to an equal division of chromatin substance, required by the chromosome theory. The problem of the participation of amitosis in the cell multiplication would be rather simple, if it were proved that the amitosis is exclusively observed in certain developmental stages and that thereby no typical mitosis oc-

curs, as was suggested by GODA in the toad's blastula. However, in sections stained by FEULGEN's nucleal method instead of GODA's water-blue eosine method, considerable number of mitotic chromosomes can be always recognized, even in the blastula, side by side with double nuclei (Plate 1, *m*). Thus one might assume that the cell multiplication is accomplished exclusively by the normal mitosis and that the so-called amitosis is only a modification of resting nuclei, having the significance of enlarging nuclear surfaces (FLEMMING, 1891).

Some authors (CHILD, 1904–'07; and GLASER, 1908) on the other hand insist that amitoses belong to the normal mode of cell division in embryonic stages because the observed frequency of the mitosis is insufficient to account for the real increases in cell number. Yet the factual data for their arguments seem to be far from convincing. In most papers the discussion has been based upon mitotic indices and partial cell numbers in some ill-defined embryonic portions, so that the possibility of cell migration from or to other parts of the body could not be thoroughly eliminated. Indeed, as was urged by BOVERI (1907), most of the observations on amitosis have even failed to demonstrate that the two nuclei of binucleate cells actually result from division of a nucleus and not from accidental union of two single nuclei.

In this paper the author intends to give somewhat more careful discussions, using a complete set of data concerning not only the total cell number and the mitotic index, but also the nuclear size and the actual duration of mitotic and intermitotic phases, etc., in successive stages of development in *Bufo* eggs. The result confirms, at least from the cytometrical viewpoint, that observed mitotic indices are really insufficient for accounting for the magnitude of cell multiplications during the course of gastrulation.

Before going further, the author wishes to express his hearty thanks to Dr. T. HAMA of the Keiô University, Prof. H. HASEGAWA of the Yamanashi University, Dr. M. USUI of the Tokyo Educational University, and to Prof. T. YAMADA of the Nagoya University for their constant encouragements and for their precious criticisms.

II. OCCURRENCE AND MORPHOLOGY OF THE DOUBLE NUCLEUS

From the first cleavage to the early blastula (stage 7 by BABA-

OKADA,* 1932), hardly any double nucleus is observed and the main part of cells is actively participating in mitosis. Although there is an obvious tendency to the synchronism in the mitotic activity, the level of the mitotic index is kept at 80%, since up to the blastula most of the "resting cells" contain slender prophase chromosomes. At the late blastula (stage 9–10) most nuclei appear somewhat vacuolated and more than 10% of them are typical double nuclei: two small spherical nuclei are attached side by side in a single cell body. The partners may be equal (Plate 1, *a*) or unequal in size (Plate 1, *b*) and sometimes they may be three in numbers (Plate 1, *c* and *d*). Cells with dumb-bell-shaped nuclei (Plate 1, *e*), with distinctly separated partners (Plate 1, *f*), or those at the moment of cytokinesis (Plate 1, *g*) are only rarely found. At the late blastula, the distribution of chromatin within each partner displays some signs of polarity: contorted masses of fine chromatin threads are lying face to face in the peripheral portion of each partner leaving behind almost transparent space. In such cases, it seems that the rupture of the nuclear membrane in the normal mitosis might be inhibited. During the gastrulation, however, most of the ectodermal cells contain vacuolated nuclei and more than 15% of them are typical double nuclei. The chromatin granules in the double nucleus are then evenly distributed (Plate 1). In *Bufo vulgaris*, the mitotic index does not exceed 10% during the gastrulation (Table 4, Section V, p. 17). The values obtained by the present author are markedly lower than those of BRAGG's measurement (1938) in *Bufo cognatus*, where double nuclei are not described.

In the neural plate stage (st. 16) cells with double nuclei can hardly be found. We are now confronted with the following question: Does the double nucleus fuse to form a single nucleus or segregate into two mono-nucleate daughter cells during the later phase of gastrulation?

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* The correspondence with SHUMWAY's normal stages (for *Rana pipiens*) is roughly as follows:

BABA-OKADA	10	(10+)	11	12	13	14	15	16
SHUMWAY	9		10		11		12	13

At the intervening stage 10+ the very first sign of invagination is detected by histological observation.

III. Measurement of Nuclear Volume

To answer the question above mentioned or to get some idea on the origin of double nucleus, mere morphological observations on microscopical sections are utterly insufficient. In this section are studied the frequency distribution curves of volumes of various types of nuclei: single nucleus, each partner of the double nucleus, and sum of partners of a double nucleus.

The measurement of the nuclear volumes was made upon central optical sections of about 100 to 200 intact nuclei. The areas were drawn on a paper by means of ABBE's apparatus and on each nucleus the major axis a and the minor axis b of the idealized ellips were determined in mm. The grouping of the nuclear volumes was conveniently performed by classifying the calculated value of $\sqrt[3]{ab^2}$ in mm. The sum of volumes of partners in a double nucleus can be similarly treated by the value of $\sqrt[3]{a_1 b_1^2 + a_2 b_2^2}$. If suitable numerical tables are previously prepared, the whole procedures can be carried out with little efforts. If needed, the absolute value of the nuclear volume can be obtained on the assumption that it is an ellipsoid according to the formula,

$$V = \pi a b^2 / 6 f^3 \quad \text{mm.}^3,$$

where f is a linear magnification of the drawing.

Table 1 a and b show percentage distributions of the volume of single nucleus, the sum of volumes of the partners of a double nucleus, and the volume of each partner in the ectoderm of various developmental stages. The results are classified under two groups, Type Y (Table 1 a) and Type O (Table 1 b), according to the range of the total volume of embryos. That the total volume is an important factor for various quantitative characters in the cell-architecture of the embryonic body has been indicated earlier (SIRAKAMI, 1952 b, c).

Type Y embryos, giant forms, come from Yamanashi prefecture. The total volume of fixed specimen is generally over 3.5 mm.^{3} Type O embryos originate from Otsuka Botanical Garden in Tokyo, their volumes ranging from 2.5 to 3.5 mm.^{3} Dwarf embryos, Type M, with volume less than 2.5 mm.^{3}, are occasionally found in Otsuka with Type O forms. It should be again emphasized here that the cell-architecture of embryos is not correlated with the habitat but with the total volume of embryos. For instance the Type Y embryos from Otsuka display quantitative characters same as those of Type Y embryos from Yamanashi prefecture.

The tables show clearly that the volume of single and double

Blastula (st. 9/10)	Sing Sum Eacl
Late blastula (st. 10)	Sing Sum Eacl
Early crescent blastopore (st. 11)	Sing Sum Eacl
Do. (st. 11/12)	Sing Sum Eacl
Crescent blastopore (st. 12)	Sing Sum Eacl
Do. (st. 13)	Sing Sum Eacl
Yolk plug (st. 14)	Sing Sum Eacl
End of gastrulation (st. 15)	Sing

* Expressed

Volumes of t
from an embryo a
Note that the vo
numbers in parer

Table 1 *b*

Total Volume of embryos: over 3.5 mm.^{3} Type Y

	Radius*	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12.0	12.5	13.0	13.5	14.0	Mean volume (μ^3)
	Volume (μ^3)	80	120	168	232	304	400	510	630	776	944	1130	1340	1580	1840	2140	2450	2800	3180	3600	4040	4510	5060	
Blastula (st. 9/10)	Single nucleus									0	2	5	10	16	22	29	23	19	7	5	4	1	1	2260
	Sum of double nucleus							0	3	0	5	5	20	18	14	11	4	3	2	1	0			1690
	Each partner of double nucleus	0	3	4	8	17	12	18	20	38	26	7	11	7	7	7	2	2	1	0				800
Late blastula (st. 10)	Single nucleus									0	3	6	16	27	29	10	1	1	0					1570
	Sum of double nucleus								0	3	2	4	4	1	1	0								1170
	Each partner of double nucleus				0	4	6	4	8	6	0	2	0											590
Early crescent blastopore (st. 11)	Single nucleus									0	5	7	10	19	34	17	12	3	0	1	0			1820
	Sum of double nucleus									0	2	5	1	11	9	8	1	1	0					1720
	Each partner of double nucleus	0	1	2	0	7	4	15	10	10	12	10	3	3	1	0								760
Do. (st. 11/12)	Single nucleus						0	4	4	18	26	16	10	2	0									970
	Sum of double nucleus				0	1	2	3	3	12	14	2	2	0										820
	Each partner of double nucleus	0	6	2	9	18	17	15	5	4	2	0												400
Crescent blastopore (st. 12)	Single nucleus						0	5	8	17	20	14	2	2	0									900
	Sum of double nucleus						0	5	5	14	6	1	1	0										770
	Each partner of double nucleus	1	0	7	7	15	21	11	3	0	1	0												370
Do. (st. 13)	Single nucleus					0	3	13	37	30	18	6	0											730
	Sum of double nucleus				0	1	1	2	3	8	2	1	0											710
	Each partner of double nucleus	0	3	2	4	11	8	6	0	2	0													350
Yolk plug (st. 14)	Single nucleus				0	1	14	32	28	16	5	2	0											600
	Sum of double nucleus			0	1	4	3	5	3	2	0													480
	Each partner of double nucleus	2	7	10	4	8	2	2	1	0														240
End of gastrulation (st. 15)	Single nucleus			0	2	15	41	45	18	3	0													470

* Expressed in an arbitary unit; 1 unit corresponds to 0.67 μ.

Volumes of three types of nucleus, those of the single nucleus, those of the sum of the double nucleus, and those of each partner of the double nucleus from an embryo are listed for each stage. The result is classified into two groups, type O and type Y, having different ranges of total embryonic volume. Note that the volume of nucleus in earlier stages is larger in larger forms. Such differences vanish when the embryo attains the yolk plug stage. Stage numbers in parentheses after BABA-OKADA (1932).

Table 1. Frequency distribution of the nuclear volumes at various developmental stages

Table 1 *a*

Total Volume of embryos: 2.5–3.5 mm.³ Type O.

	Radius*	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	10.5	11.0	11.5	12.0	Mean volume (μ^3)
	Volume (μ^3)	80	120	168	232	304	400	510	630	776	944	1130	1340	1580	1840	2140	2450	2800	3180	
Early blastula (st. 8)	Single nucleus										0	2	16	26	12	11	5	2	0	1730
	Sum of double nucleus										0	1	2	1	0	2	2	0		1820
	Each partner of double nucleus					0	3	1	5	3	4	4	1	1	0					840
Blastula (st. 9)	Single nucleus						0	1	3	5	30	29	26	13	3	2	0			1180
	Sum of double nucleus							0	2	2	7	1	3	1	1	0				990
	Each partner of double nucleus			0	5	3	7	5	6	4	1	1	0							505
Late blastula (st. 10)	Single nucleus						0	1	12	23	32	24	30	13	2	0				1060
	Sum of double nucleus								0	4	4	1	1	0						935
	Each partner of double nucleus		0	1	1	2	4	1	3	2	0									465
Early crescent stage (st. 12)	Single nucleus					0	1	6	15	35	29	14	6	0						860
	Sum of double nucleus							0	3	6	0									730
	Each partner of double nucleus		0	1	1	8	5	3	0											350
Crescent blastopore (st. 13)	Single nucleus				0	3	9	13	36	22	16	3	0							680
	Sum of double nucleus				0	1	2	4	5	4	0	1	0							610
	Each partner of double nucleus	0	2	6	7	8	9	1	0	1	0									300
Yolk plug stage (st. 14)	Single nucleus				0	6	15	37	28	20	18	0								585
	Sum of doudle nucleus				0	4	0	1	1	0										400
	Each partner of double nucleus	0	5	5	1	0	1	0												170
End of gastrulation (st. 15)	Single nucleus		0	1	11	42	37	20	6	0										400

Table 1 *b*

Total Volume of embryos: over 3.5 mm.³ Type Y

nuclei displays a unimodal (V-shaped) distribution curve and the range of fluctuation falls closely together. The results might exclude the following two possibilities: (1) A double nucleus may be formed by an accidental union of two single nuclei (BOVERI, 1907); (2) A single nucleus may transform into a double nucleus only when it grows up twice as large as the normal size.

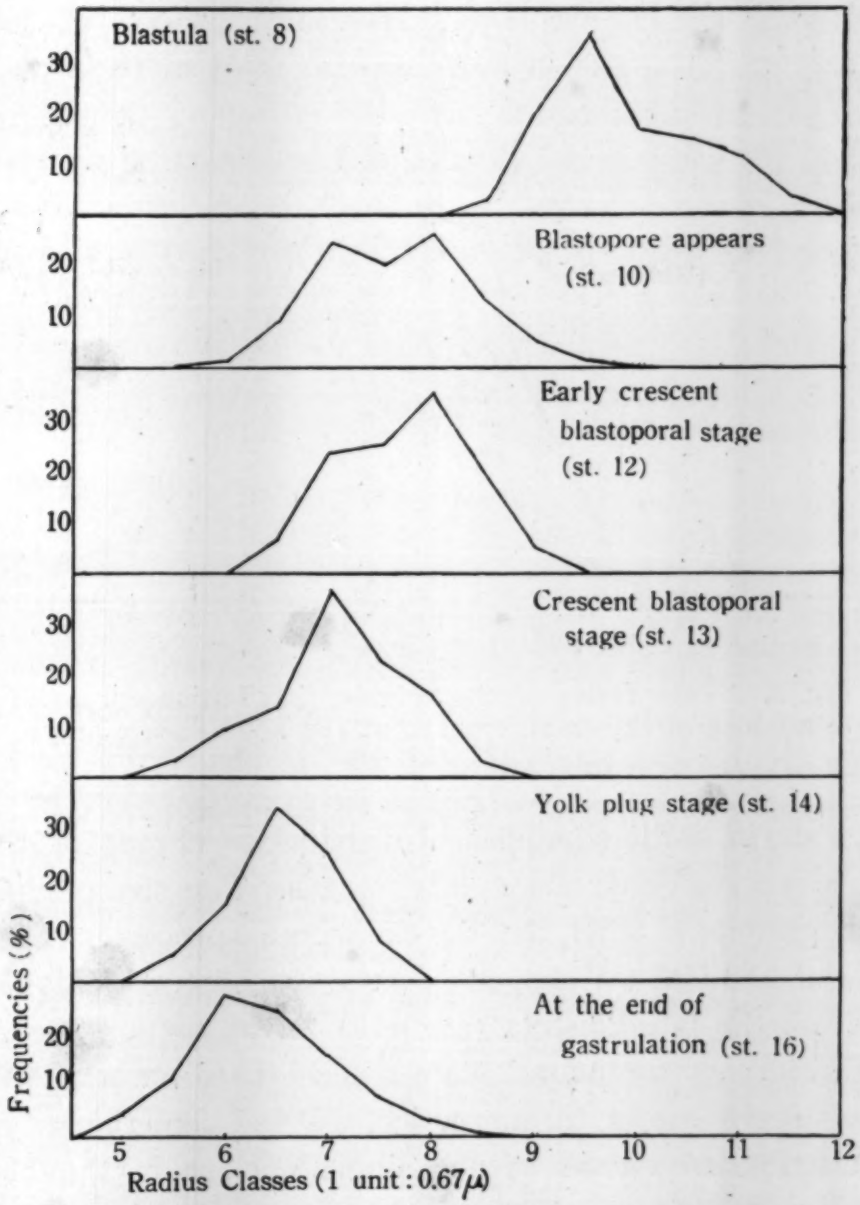


Fig. 1. Percentage distribution curves of the volume of single nuclei in the ectodermal region in normal development. Figures in parentheses are stage numbers by BABA-OKADA. Note that the radius decreases gradually and the W-shaped distribution curve is observed at the beginning of the gastrulation.

To go a step further, if the double nucleus is rapidly dividing into two single nuclei, the shape of the distribution curves should be two-peaked (W-shaped), because average volume of the partners in a double nucleus is usually only a half of the ordinary single nucleus (Table 1). Such shapes of the distribution curve are really observed at the beginning and at the end of gastrulation (Fig. 1 and 2).

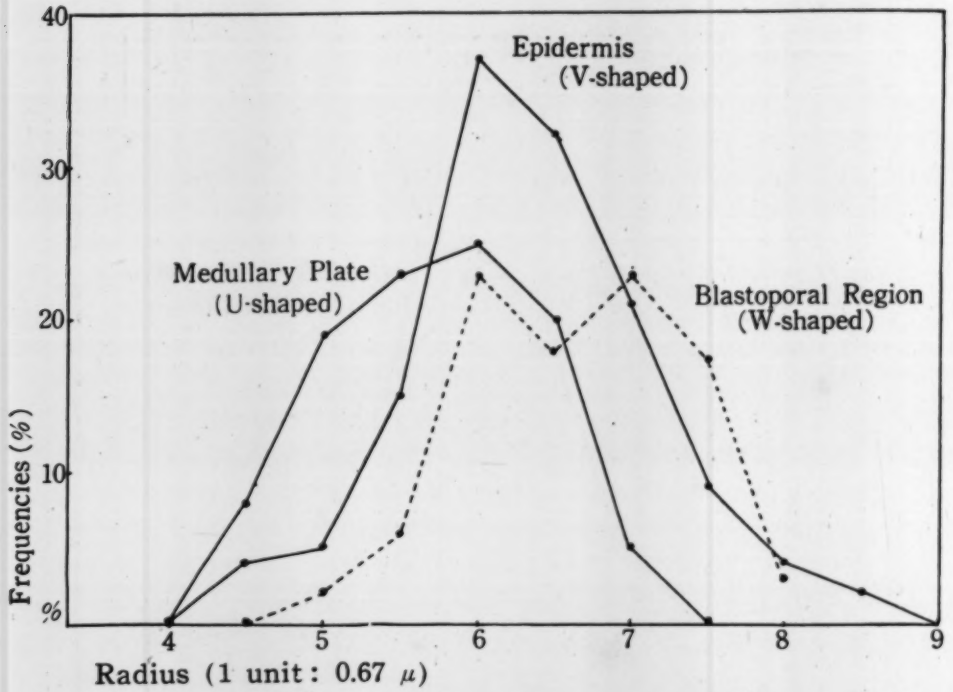


Fig. 2. Three forms of the volume distribution curves of the single nucleus in the same embryo at the neural plate stage.

Fig. 1 shows some examples of distribution curves of the single nucleus in successive developmental stages. The size of the nucleus decreases gradually with the developmental process. Obtuse (U-shaped) and bimodal (W-shaped) curves are observed in the normal embryo from the late blastula (st. 9–10) to the early phase of gastrulation (st. 11). At the neural plate stage (st. 16), where the double nucleus scarcely comes to our notice, we find sometimes typical W-shaped distribution curves of the single nucleus in the blastoporal region (Fig. 2). After this critical period double nuclei vanish from every part of the embryonic body.

Incidentally it may be noted that the size of nucleus varies according to the volume types of embryo at the beginning of development, as indicated in

Table 1. After the appearance of the blastopore, the size differences of nucleus vanishes gradually. This finding is also supported by many unpublished data.

IV. Counting of the Total Cell Numbers

To discuss the participation of double nuclei in the course of cell multiplication one must, first of all, get perfect sets of data concerning the increase of cell number in various embryonic parts during successive developmental stages. Total cell counts were attempted by Bragg (1938) in *Bufo cognatus* and by Sze (1953) in *Rana pipiens*, but their results comprise too little information on the individual fluctuations to be treated systematically, so that our knowlege on this subject remains very poor. This might be a fundamental weak point accompanying almost all the arguments hitherto proposed for the above and analogous problems. Some discussions are based upon nuclear densities instead of total cell numbers (Herlitzka, 1897; Glaser, 1914; McKeehan, 1951). But this method is methodologically not correct enough, since the possibility of the cell migration can not be excluded.

A. Methods

The method for counting cell numbers adopted earlier by the present author (Sirakami, 1952 *a*, *b*) is very plain in principle but rather laborious in practice.

Embryos were fixed in 10% formalin and cut into perfect serial sections, 10 μ in thickness, by ordinary paraffine method. The cell number was estimated by counting nuclei in every part of serial section of the embryos. The accuracy of counting depends upon the method of staining of the nucleus, which is usually difficult on account of the inevitable by-staining of yolk granules. The following modification of Goda's water-blue eosine method was found most suitable for the selective staining, especially when formalin was used as the fixative.

Water-blue	0.005%	200 cc.
Picric acid, saturated solution		5 to 20 cc.

The measurement of the nucleus number consists in the following two procedures: the determination of the area of cross sections of

various parts in the section, and the counting of nuclei in an unit area for each part. The areas of various parts were estimated by weighing cut-out drawings on the paper, the weight of which is previously determined in terms of gram per square centimeter. Distribution densities of the nucleus were determined by counting nuclei in a field of the known area by CHALKLEY's circle ring method or by projection of black squares by means of ABBE's apparatus. Since most of the nuclei were cut into several sections, the observed density C_0 in one section may be overestimated. The true density C was calculated by a formula,

$$C = C_0/(1 + d/D), \tag{1}$$

where d is the average depth of nuclei, *i.e.* the thickness of nucleus measured in the direction normal to the plane of sections, and D the thickness of the section. Of course the depth of the nucleus does not appear in the section, but it can be substituted by the average maximal diameter of the almost spherical nuclei. Fixation with formalin causes spherical condensation of nucleus and is found to be suitable for mathematical adjustment by means of formula (1). The number of nuclei N in one part of the section was thus obtained by the following formula,

$$N = S \times C = S \times C_0/(1 + d/D), \tag{2}$$

where S is the area of that part.

The total number of cells of an embryo was estimated by summing up these values of N through the whole series of sections.

In practice, the procedure was simplified by performing the measurements at a certain interval, and the values were treated as the mean values for that interval.

The reliability or the error of this method depends upon many factors: the magnification of the drawing, the homogeneity of the paper, the size and shape of the embryonic parts, the regularity of the thickness of sections, etc. However, in principle, if we control the error of each nuclear number, calculated by the formula (2), the error of the total count is automatically controlled.

As the formula indicates, a nuclear number N is composed of many factors, each of them having its proper range of error, which

is conveniently treated by the coefficient of variation (100× standard deviation/mean value %), namely by V_N, V_C, V_S, V_d, and V_D respectively.

Using the idea of approximation of compound error, the ultimate error V_N of the nuclear number was calculated as follows:

The variance of N,

$$\sigma_N^2 \fallingdotseq (\partial N/\partial S)^2\sigma_S^2 + (\partial N/\partial C)^2\sigma_C^2 + (\partial N/\partial d)^2\sigma_d^2 + (\partial N/\partial D)^2\sigma_D^2.$$

While $V_N = \sigma_N/N$ in definition, we obtain

$$V_N = \sqrt[2]{V_S^2 + V_C^2 + (V_d^2 + V_D^2)(d/D)^2(1+d/D)^{-2}}.$$

Assuming then $d \fallingdotseq D$ or $1 + d/D \fallingdotseq 2$,

$$V_N = \sqrt[2]{V_S^2 + V_C^2 + (V_d^2 + V_D^2)/4}. \qquad (3)$$

Consulting the results of careful measurements on the whole sections of some embryos, we estimated the following allowance of the range of errors.

1. The distribution density C of the nucleus in a germ layer deviates widely with depths from the embryonic surfaces. Therefore, when we take one germ layer as a whole, the error V_C becomes hopelessly large. But, if the area is divided into several strata of similar nuclear densities, V_C can be kept at less than $\pm 15\%$.

2. The error in measurements of the area S depends upon the size and shape of drawings. When a part is magnified to over 10 cm.^{2}, the error diminishes at least to the level of the homogeneity of paper ($\pm 2\%$ or less). Small cross sections of the neural tube can be thus measured with the same accuracy, if they are drawn in higher magnification. However, when the measurements are made for instance on every five sections, the value of V_S can not be lower than $\pm 5\%$.

3. The fluctuation of the thickness of the section is assumed to be $\pm 10\%$.

4. Many measurements of the nuclear diameters suggest that the coefficient of variation V_d is less than $\pm 10\%$.

Putting these ranges of errors into the formula (3), we obtain *ca.* $\pm 20\%$ as an estimate of the composed error.

In a similar manner, we can calculate the volume of various em-

bryonic parts, summing up the value $S \times D$, with their error calculated by a formula,

$$V_V = \sqrt{V_S^2 + V_D^2} = \pm 10\%$$

B. Remarks on the cell number and the cell-architecture

The result of our cell counts is summarized in Table 2. It is clearly indicated that the cell number as well as the volume of each part of embryos vary widely according to their total volume, even if we take the same developmental stage. But if the embryos are classified into several volume types (Type Y and O, in the present Table), they show considerably regular cell-architectures (see section II and SIRAKAMI, 1952 *b*).

Microscopical studies show that the embryo at the morula stage is composed of three main portions, which are easily distinguishable from one another. The animal area is a deeply pigmented portion, which extends from the animal pole to the lateral wall of the blastocoel. The marginal zone forms a ring beneath the animal area, encircling the spherical egg near the equator. The latter part is distinguished from the former by its poor pigmentation, and from the remaining vegetal area by its smaller yolk granules. Although such organization is well known, so far as we know little attempt has been made to clarify the relation of volume percentage of these portions during the course of further development.

Measurements of the volume percentage of various embryonic portions in three successive developmental phases (*A*, *B*, and *C*) are given in Table 3.

As is shown in Table 3 *A*, the volume percentage of animal area plus marginal zone in the morula to blastula stage fluctuates from 59% to 37%, whereas the total volume fluctuates from 2.18 mm.^{3} to 6.18 mm.^{3} (*i.e.* about 2.84 times). Therefore, the actual volume does not change strictly in proportion to the total embryonic volume. On the other hand, if the actual volume of that portion is kept constant regardless of the total volume of embryos, the percentage should decrease from 59% to about 21% (*i.e.* 1/2.84 times). It might be therefore concluded that the actual volume of the animal area plus marginal zone really increases with the total embryonic volumes, but not proportionately, showing a tendency toward a constancy of the actual volume.

Table 2. Cell numbers and volumes of different parts in the developing *Bufo* embryo

A. Medium size embryos (type O): total volume 2.5–3.5 mm.^{3}

Stage and Embryo number	Cell number (unit: 10^4)					Volume (unit: mm.^{3})					Volume ratio* (unit: 10^{-7})
	Multiplicating part		Entoderm	Total		Multiplicating part		Entoderm	Total		
	Ectoderm	Mesoderm				Ectoderm	Mesoderm				
8 f	1.5	—	—	1.2	2.7	0.75	—	—	1.85	2.60	192
10 Y	3.29	—	—	0.16	3.45	1.73	—	—	1.00	2.73	192
10 E	3.74	—	—	0.85	4.59	1.55	—	—	1.20	2.75	151
10+ g	2.4	—	—	0.6	3.0	0.75	—	—	1.50	2.25	122
10+ B	5.09	—	—	0.22	5.31	1.58	—	—	1.00	2.58	120
10+ h	4.06	—	—	0.12	4.18	1.55	—	—	1.19	2.74	139
11 A	3.3	—	—	1.5	4.8	0.82	—	—	2.26	3.08	80
12 E	4.6	—	—	1.6	6.2	0.75	—	—	1.35	2.10	78
	Epi-dermis	Neural part				Epi-dermis	Neural part				
13/14 F	6.3	—	5.3	1.6	13.2	0.72	—	0.92	1.49	3.13	46
13/14 B	6.9	—	5.8	1.2	13.9	0.78	—	1.12	1.20	3.10	45
13/14 A	7.2	—	5.9	1.0	14.1	0.74	—	1.10	1.09	2.93	48
15 B	7.8	3.2	5.7	2.2	18.9	0.78	0.29	1.03	1.37	3.47	36
16 F	4.1	6.7	3.8	3.2	17.8	0.32	0.45	0.57	2.02	3.36	28

B. Large size embryos (type Y): total volume over 3.5 mm.^{3}

Stage and Embryo number	Cell number (unit: 10^4)					Volume (unit: mm.^{3})					Volume ratio* (unit: 10^{-7})
	Multiplicating part		Entoderm	Total		Multiplicating part		Entoderm	Total		
	Ectoderm	Mesoderm				Ectoderm	Mesoderm				
8 e	0.6	—	—	0.2	0.8	1.55	—	—	4.35	5.90	438
10+ K	1.4	—	—	0.9	2.3	0.9	—	—	3.4	4.3	149
10+ T	2.64	—	—	—	—	2.58	—	—	4.38	6.96	140
10+ R	3.0	—	—	0.3	3.3	2.42	—	—	4.10	6.52	124
10+ F	3.0	—	—	1.2	4.2	1.1	—	—	4.0	5.1	72
12 A	5.0	—	—	2.5	7.5	1.25	—	—	4.20	5.45	46
12/13 e	7.9	—	—	1.3	9.2	1.85	—	—	5.15	7.00	33
	Epi-dermis	Neural part				Epi-dermis	Neural part				
13 a	9.8	—	2.3	2.1	14.2	1.90	—	0.5	4.2	6.60	30
14 R	11.6	—	4.2	0.8	16.6	2.3	—	1.55	4.55	8.40	29
15 p	11.0	—	1.7	3.0	15.7	1.2	—	1.0	3.7	5.9	29
15 h	8.0	—	1.1	3.3	12.4	0.9	—	1.2	3.9	6.0	39
16 E	7.1	5.2	6.5	3.4	22.2	0.56	0.41	0.90	1.94	3.81	26

* The ratio of the mean cell volume of the multiplicating part to the total embryonic volume (see p. 13–14).

The data are classified into two volume types.

Table 3. The volume percentage of embryonic portions to the total embryonic volumes in three successive developmental phases

A. Morula to blastula

Embryo No.	Total volume of embryo mm.³	Animal area %	Animal area plus marginal zone %	Yolk cell mass %
7* M	2.18	26	59	41
3 F	2.44	29	61	39
3 G	2.47	30	57	43
5 B	2.56	28	50	50
5 a	2.58	28	50	50
5/6 A	5.63	25	38	62
6/7 A	6.18	23	37	63

B. Beginning of the gastrulation

Embryo No.	Total volume of embryo mm.³	Ectodermal part %	Total multiplicating part %	Yolk cell mass %
10* g	2.25	33	—	—
10 B	2.58	39	62	38
8 f	2.60	29	—	—
10 Y	2.73	36	63	37
10 h	2.75	30	57	43
10 E	2.75	30	57	43
11 A	3.08	27	—	—
10 K	4.3	21	—	—
10 F	5.1	22	—	—
10 R	6.52	22	37	63
10 T	6.96	23	37	63

C. After the gastrulation

Embryo No.	Total volume mm.³	Ectodermal part %	Total multiplicating part %	Yolk cell mass %
15* d	2.48	40	61	39
20 D	2.65	26	45	55
16 F	3.36	23	40	60
16 E	3.81	27	51	49
14 g	4.39	28	78	22
20 A	4.44	16	41	59
20 h	4.7	13	43	57
15 h	6.0	15	35	65
18 a	8.1	19	43	57

* Stage numbers by BABA-OKADA.

For explanations see the text.

As the development proceeds from the blastula to the gastrula, we can still distinguish three morphological portions by their cell size, pigmentation, and other cytoplasmic inclusions. As shown in Table 3 *B*, the part of the embryo, which corresponds morphologically to the animal area plus marginal zone in the morula to blastula, occupies about 60% to 40% of the total embryonic volumes, fairely corresponding also volumetrically to the above mentioned portion in the early developmental phase of the embryo with similar total volumes.

Analogous volumetric relations persist also in the embryos of still later stages (Table 3 *C*), where this part is differentiated into epidermis, neural areas, and mesoderm.

If we take the embryo of similar total volumes, the volumetric percentage of the animal area plus marginal zone to the total embryo remains constant even in these periods, suggesting that there is no tendency of its growth at the expense of yolk cell masses. So we might call this volumetrically independent part "multiplicating part," in which cells may repeatedly divide, getting smaller and smaller in size.

Just in this part we can discuss the kinetics of cell multiplication, because here the possibility of large scale cell migrations into or from other part of the embryo is excluded. The first difficulty to be overcome was the fluctuation of cell number in the multiplicating part according to total embryonic volumes. How is it possible to draw *an* ideal curve of the cell multiplication, using data from embryos of various total volumes?

Figure 3, in which the ratio of the average cell volume in the multiplicating part to the total embryonic volume (volume of the multiplicating part/number of cells in the multiplicating part/total embryonic volume) is plotted against exact time scale,* suggests such a possibility of eliminating volume fluctuations in various embroys. The ratio calculated from the data given in Table 2 decreases regularly as the embryo develops and shows a tendency to form a single curve in spite of the wide divergencies in the total embryonic volumes. When the value is over 200×10^{-7}, that is, when the mean volume of

* Since there is no distinct correlation between the embryonic size and the developmental velocity (SIRAKAMI, 1955), we adopted a single standard time scale at 20° C. for both types of the embryo.

cells in the multiplicating part is larger than 50,000th of the total embryonic volume no blastopore is to be seen. The gastrulation begins (st. 10+) in every type of embryos when the ratio is lowered from this critical value, and at the end of gastrulation the value falls to about 30×10^{-7}. If the volume growth is to be neglected in these periods, the value should be reciprocally proportional to the cell number in the multiplicating part. Accordingly, the curve can be regarded as a reciprocal multiplication curve of the embryonic cells during the course of gastrulation.

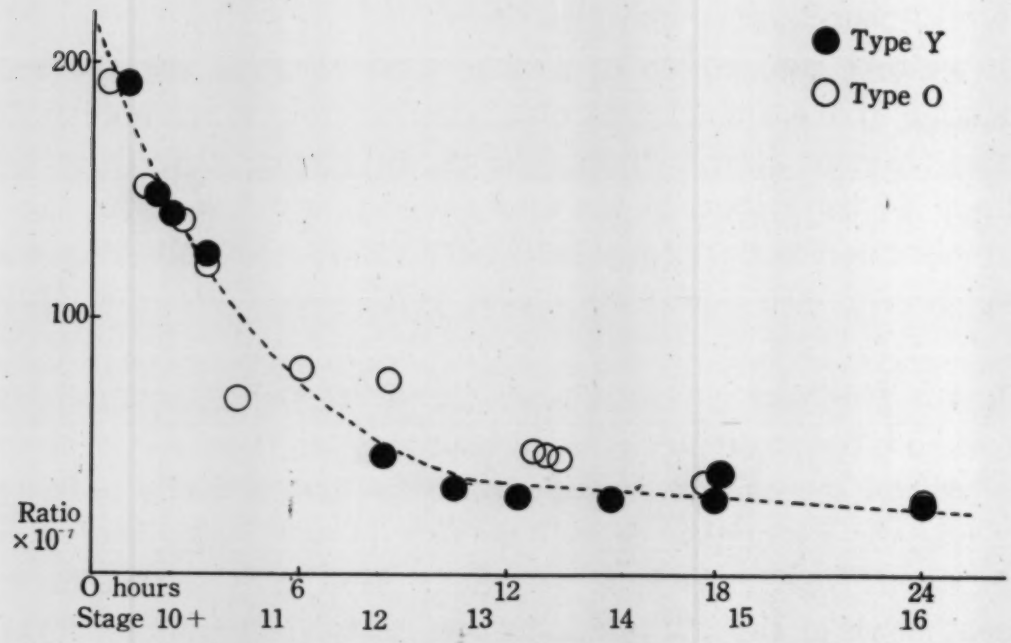


Fig. 3. Reciprocal cell multiplication curve during the early developmental stages of *Bufo vulgaris*. The ratio of mean cell volume of the multiplicating part to the total volume (abscissa) is plotted against standard time scale (ordinate). For further explanations, see the text.

V. Relation of the Mitotic Index to the Kinetics of Cell Multiplication

The curve in Fig. 3 indicates that in the idealized case the cell number of the multiplicating part is expected to increase about 5-fold during the course of gastrulation, which lasts for about 20 hours at 20° C. In our measurements (Table 2) the mean cell number of the ectoderm plus marginal zone at the beginning of gastrulation (st. 10+) is 37,000 for the medium sized embryos (Type O) and about 25,000 for larger forms (Type Y). At the end of next nine hours, when

the embryo attains crescent blastopore stage (st. 13), more than 100,000 cells are present in the same area. Does such an intensive cell multiplication depend solely upon usual mitoses? To answer this question it is necessary to establish the relation of mitotic index and duration of mitosis to the numerical course of cell multiplication.

Let an initial cell number in a definite part of the embryonic body be N_0 and the fraction of cells in mitosis be a_1, the resulting cell number N_1 after the lapse of average mitotic duration t_m will be:

$$N_1 = N_0(1 + a_1). \tag{4}$$

At this time some other cells may have entered into mitosis and if we put their index at a_2, then the cell number N_2 after another average mitotic duration t'_m will be:

$$N_2 = N_0(1 + a_1)(1 + a_2), \tag{5}$$

and so on.

In the simplest case, where namely the values of mitotic duration t_m and the mitotic index a_i remain constant throughout the whole period T, the final cell number N can be expressed:

$$N = N_0(1 + a_i)^{T/t_m}. \tag{6}$$

From these considerations it is evident that the mitotic index alone can not account for the whole process of cell multiplication. If t_m is very small, a very steep increase in cell number can be explained by a low mitotic index. On the contrary, when only a slow increase is recorded with very high mitotic index, we are allowed to conclude that the whole mitotic process advances only sluggishly.

Similarly, if we get serial values of cell numbers, N_0, N_1, N_2, etc., increasing stepwise after each average mitotic duration (the duration may not be necessarily constant for each step), we can calculate a series of expected values of mitotic indices for each step (Table 4) on the assumption that the cell multiplication is taking place exclusively by regular mitosis.

In practice the procedures are as follows: we first draw an ideal reciprocal multiplication curve (Fig. 3) along the exact time scale in the ordinate, then we cut out the curve with distances of average mitotic duration, which have been obtained by other measurement. If we calculate the ratio of R_i/R_{i+1} from a pair of neighbouring

heights of the reciprocal multiplication curve, the results give us the value of N_{i+1}/N_i and eventually the value of a_i (*cf.* Formula 4).

In such procedures, we should not forget a fundamental postulate that all the cells must belong to a well-defined "cell-cone" (WOODGER, 1933), in other words, cells must have descended from a common "apical cell" (WOODGER) exclusively through division, and that the migration of cells from or to other cell-cones is perfectly excluded. The substitution of N_i by a value of cell *density* found in some microscopical sections would make the calculation meaningless.*

It is generally difficult or practically impossible to obtain the series of cell numbers N_0, N_1, N_2, etc., fulfilling such requirements. However in the present studies on the early stage embryo, we are fortunately allowed to apply this method on a definite multiplicating part in the animal hemisphere. As has been explained in the preceding section, cells in the multiplicating part belong to a cell-cone. Accordingly, if we find the most probable value of mitotic duration, we can calculate the expected value of mitotic indices and compare them with observed ones.

It is practically impossible to get exact values of mitotic duration for the living embryonic cells during the early phase of gastrulation by direct observation, because cells are heavily laden with minute yolk and pigment granules.

However when we take certain developmental stages, free from double nuclei and with constant mitotic index a, we can calculate the value of t_m from the formula (6) in section V, using the value of initial cell number N_0, resulting cell number N, and the total time lapse T.

For the early cleavage stage, where
$N_0 = 4$ (micromeres at the end of the third cleavage), $N = 1.4 \times 10^4$,

---

* MCKEEHAN (1951) observed that the cell density in the lens ectoderm of the chick embryo (16 to 19 somites stage) was doubled in the course of five hours with the mitotic index of about three percent. From these values the interval of successive mitosis was calculated on the assumption that no migration occurred and was found to be 10 minutes. However, he used measurements of cell densities (instead of cell numbers) and his mathematical treatment was not free from ambiguities. His calculation does not give the value of intervals between two successive mitoses but that of mitotic durations. His estimation seemed too small even for the mitotic duration. The reason may be, as he himself suggested, that the morphogenetic movement of cell was playing an important rôle.

Table 4. Frequencies of the mitosis and the double nucleus in the ectodermal region during the normal course of the development (at about 16° C.)

	Embryo No.	Embryo type	Double nucleus %	Mitotic index %	Cells counted	Part of embryo	Expected mitotic index %
Middle blastula	8*– N	Y	10	21.0	601	Animal pole	
	8/9 w	O	12	7.2	802	〃	
	8/9 T	O	17	6.9	695	〃	
	9 A	Y	19	4.8	707	〃	
	9 e	O	9	7.3	861	〃	
	9 g	O	—	7.1	531	〃	
	10– r2	Y	12	10.3	1000	〃	
Late blastula	10 L	Y	12	6.6	1118	〃	
	10 E	Y	37	9.1	741	〃	
	10 E′	O	11	5.3	1000	〃	
	10 d	O	12	4.8	1000	〃	
	10 f	O	11	3.4	2211	〃	30
					620	Blastopore lip	
	10 e	Y	19	5.3	714	Animal pole	
			21	3.0	204	Blastopore lip	
Early gastrula	11 A	O	4	4.0	449	Animal pole	25
	12 A	Y	25	5.7	1000	〃	
	12 sp	Y	16	5.2	1000	〃	24
	12 m	Y	14	4.0	789	〃	
Middle gastrula	13 X	O	11	3.5	1250	〃	
			7	2.0	2383	External lip	
			13	1.5	543	Internal lip	
	13 m	O	10	4.1	1002	Animal pole	
			12	4.0	1052	External lip	
			17	1.3	479	Internal lip	
	13 d4	Y	11	2.5	1000	Animal pole	9
			9	3.7	1000	Blastopore lip	
	13 k	O	9	3.0	741	Animal pole	
			9	5.2	745	External lip	
			6	4.0	762	Internal lip	
	13 T	O	17	3.8	830	Blastopore lip	
			15	3.8	830	Animal pole	
Late gastrula	14 g	O	12	2.8	1000	〃	
	14 sp	Y	9	3.0	1000	Mesoderm	3
	14 N	Y	14	2.1	1000	Animal pole	
	15 a	O	5	0.8	419	〃	
			7	1.3	609	External lip	3
			7	2.1	344	Internal lip	
Early neurula	16 sp	O	2	4.4	997	Animal pole	
	16 x	O	2	4.1	1007	〃	
	16 j	O	0	1.9	746	Neural plate	
			0	0.6	696	Epidermis	
	16 h	O	0	2.5	474	Neural plate	
			0	1.2	517	Epidermis	2
	16 k	O	0	1.1	450	Neural plate	
			0	0.4	718	Epidermis	
	16 N	Y	0	1.2	467	〃	
			0	1.0	650	Neural pate	

* Stage number after BABA-OKADA.

The expected mitotic index was calculated from an ideal curve of cell multiplication and an estimated minimal mitotic duration (80 minutes). Further explanations are given in the text.

$a = 80\%$ or 0.80, $T = 19.0$ hours (22°-24° C.), we obtain the value:

$$t_m = 83 \text{ minutes.}$$

For the earlier neurula (st. 15 to 16), where
$N = N_0 \times 1.15$ (calculated from the multiplication curve)
$a = 3\%$ or 0.03, $T = 6.0$ hours (20° C.), we obtain the value:

$$t_m = 81 \text{ minutes.}$$

These values are for 20°–24° C. However in the following discussion only data for *ca.* 16° C are available. At lower temperature the mitotic duration might be longer. Thus in the following calculation the average mitotic duration was assumed to be not less than 80 minutes.

The values of mitotic duration are summarized in some recent works (HUGHES, 1952; and HOFFMAN, 1953). The value ranges from 30 to 180 minutes for higher vertebrates. JOLLY's data for the erythroblasts of *Triton* are 150 minutes at 20° C. It is generally accepted that the high rate of cell multiplication is always realized by the shortening of the intermitotic time and not by that of mitotic duration, which is assumed to be constant (MÖLLENDORFF, 1937; HOFFMAN, 1947).

Results of our measurements of the frequency of mitotic figures and double nuclei at various developmental stages are summarized in Table 4 together with expected values of the mitotic index calculated from data on the kinetics of cell multiplication during the course of gastrulation. As is clearly shown in the table, the observed mitotic indices in the earlier phases of gastrulation are distinctly inferior to the expected ones, which are calculated on the assumption that the cell multiplication is carried out exclusively by the normal mitotic division. It might be here emphasized again, that just in this period we find abundant double nuclei.

VII. DISCUSSION AND CONCLUSION

Now we come to the conclusion, that the observed mitotic indices are insufficient to account for the steep increase in cell number in the earlier phases of gastrulation, where the double nucleus is most abundantly found. Of course the conclusion is valid only when the assumptions made for the calculation are correct. As the formula (6), in section V (p. 15) suggests, if a sudden fall (to about 1/4) of our

estimated value of t_m occurs in these periods, actual processes of cell multiplication would be explained solely in terms of normal mitosis. However at the present state of our investigation we have no data indicating such a possibility.

Of course above conclusion does not immediately allow the possibility that the double nucleus participates in the normal course of cell multiplication. But the distribution curves of the volume of single nucleus and double nucleus (section III, p. 6) furnish some clues to this problem. Their ranges of volume fluctuation fall closely together. Thus BOVERI's classical idea of accidental union of two nuclei does not appear applicable. Another possibility that the cell divides into two daughter cells by the typical mitosis, which fuse again to form a syncytium, requires even steeper cell multiplication curves, which can hardly be explained by observed mitotic indices (Table 4). It should be pointed out here that the double nucleus was always regarded as *one* nucleus in our *cell* counting.

Thus we have to admit the possibility that the double nucleus plays an active rôle in the cell multiplication during the earlier phases of gastrulation.

As will be reported in the next paper, the double nucleus disappears when embryos are cultured in pure oxygen at high temperatures. Normal mitotic figures increase at the same time, probably at the expense of the double nucleus.

Accordingly it may be assumed that the double nucleus is a modification of the normal mitosis under deficient oxygen supply, compensating the latter for the rôle of cell multiplication.

SUMMARY

1. Amitosis-like "double nucleus" was described in the developing toad embryo during the course of gastrulation.

2. Distribution curves of volumes of the normal and the double nucleus were discussed in respect to the volumetric relation between both forms. The possibility of division of single nucleus into a double nucleus was suggested from quantitative data.

3. A method and the result of total cell count were given. Cells in a volumetrically independent embryonic part, corresponding to the ectoderm and mesoderm, were found to increase 5-fold in number during the course of gastrulation.

4. A quantitative relation of the mitotic index and the cell multiplication was established.

5. A calculation on the basis of data concerning the actual course of cell multiplication, frequencies of mitosis and double nucleus, and mitotic as well as intermitotic durations, showed that the observed mitotic index was really insufficient to account for the cell multiplication in the early gastrula, where the double nuclei were most abundantly found.

6. Thus the possibility of participation of the double nucleus in the cell multiplication was suggested.

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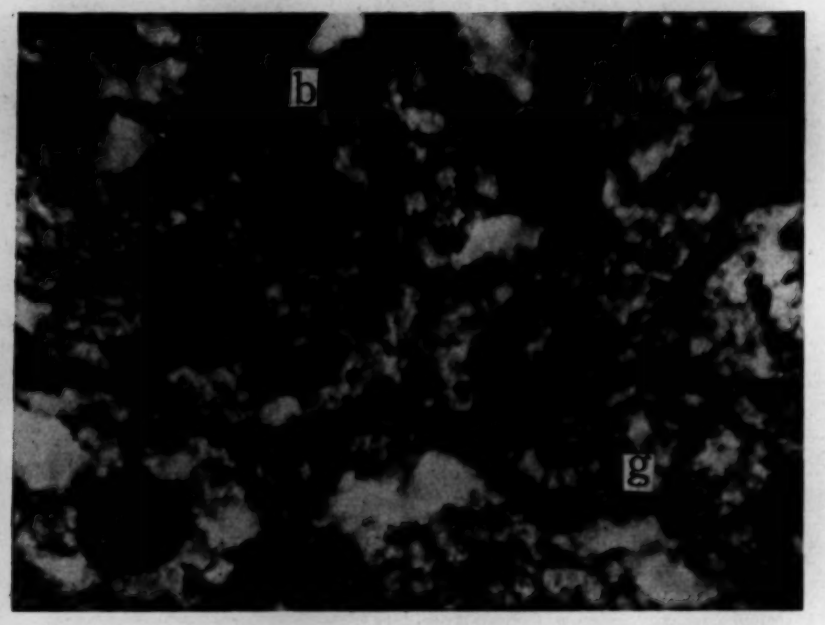
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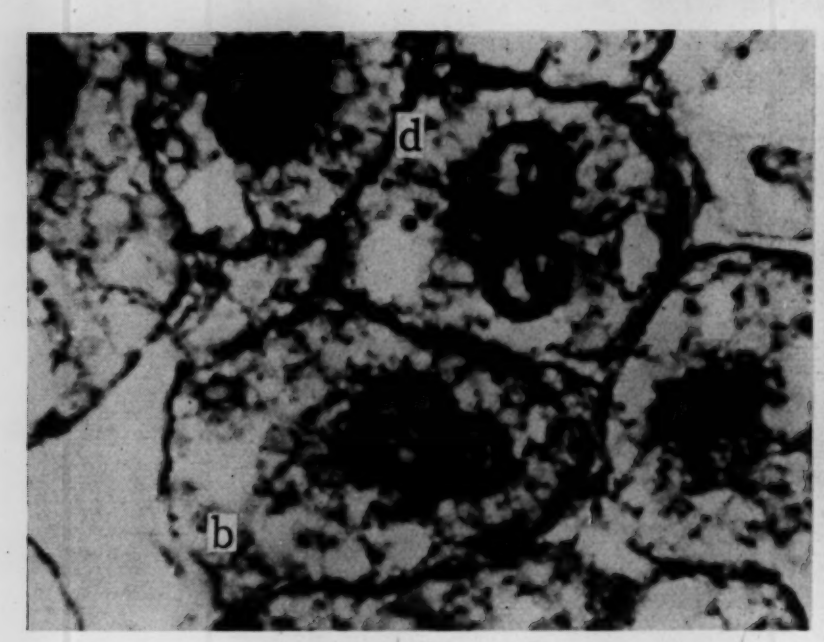
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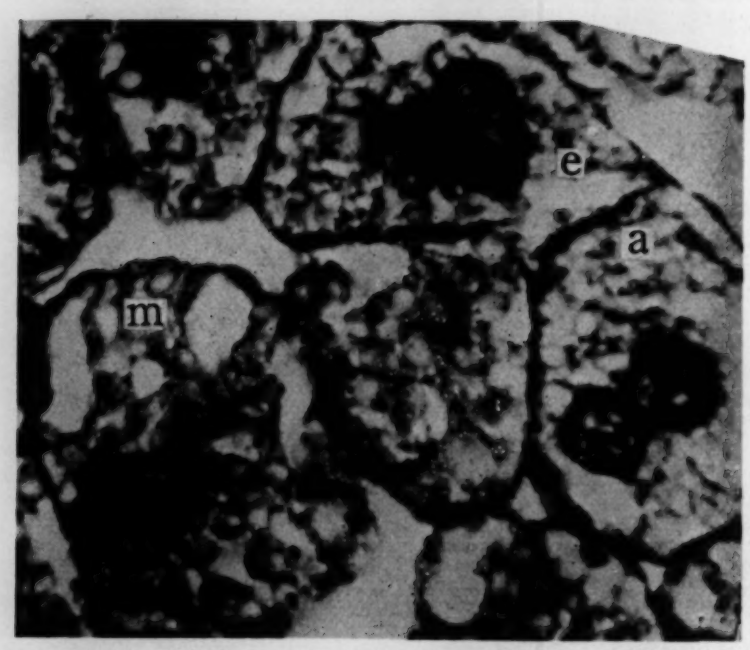
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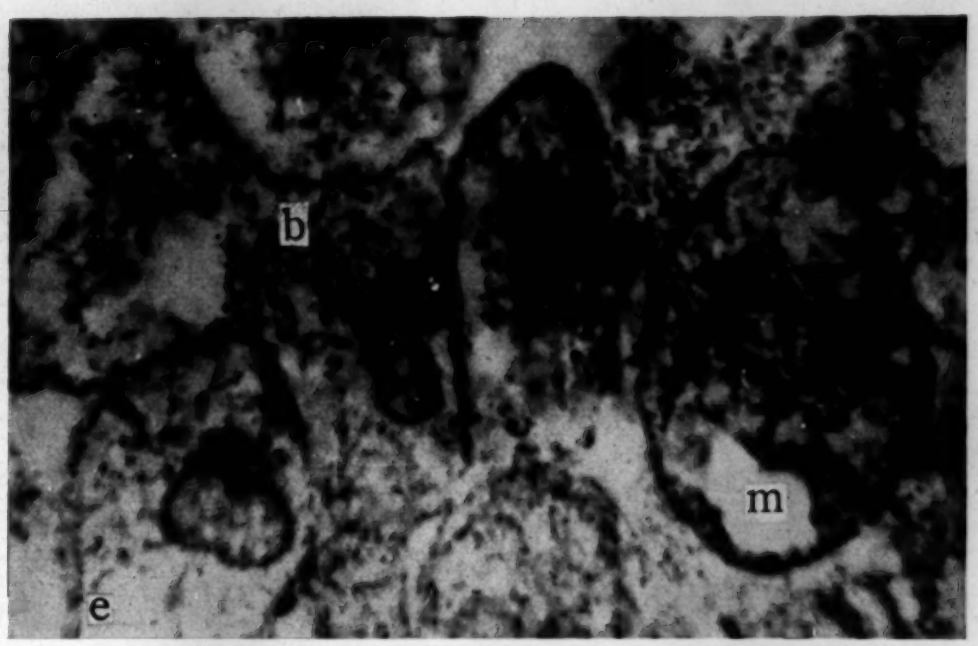
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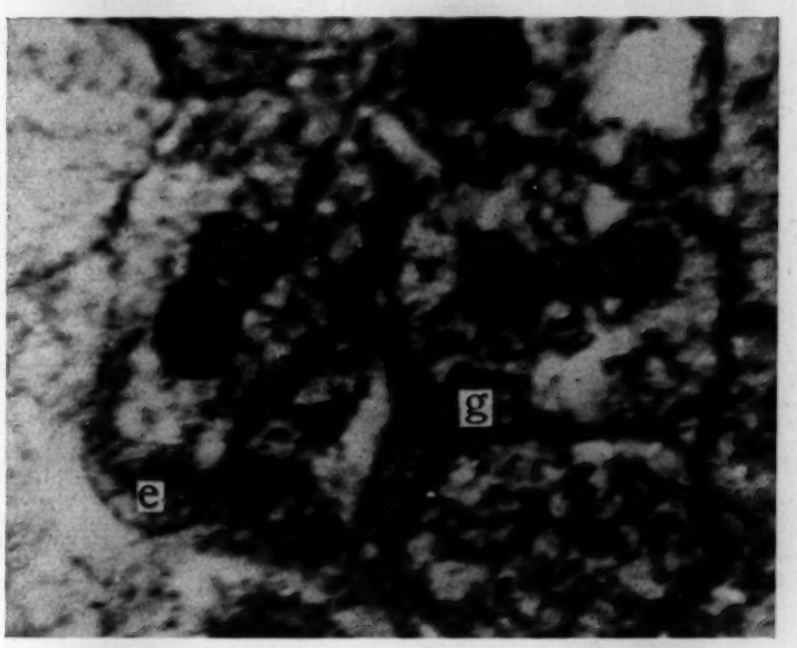
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6



7

EXPLANATIONS OF PLATE 1

Various types of the double nucleus found in the ectoderm at the crescent blastopore stage, of *Bufo vulgaris*. ×900.

Fig. 1-5. Fixed by ZENKER's fluid, stained progressively with HEIDENHAIN's hematoxylin.

FIG. 6 and 7. Fixed by BOUIN's fluid, stained with water-blue picric acid.

a : Partners are equal in size.
b : Partners are unequal in size.
c : Partners are three in number.
d : Partners are dividing into three.
e : Dumb-bell-shaped nucleus.
f : Three partners distinctly separated.
g : Probably at the moment of cytokinesis.
m: Typical mitosis.

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EXOGASTRULATION INDUCED BY CALCIUM DEFICIENCY IN THE SEA URCHIN, *PSEUDOCENTROTUS DEPRESSUS**

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The initial step of the study on the effects of various chemical agents on determination in echinoderms was taken in 1892 by HERBST when he found that the exposure of developing sea urchin embryos to lithium salts resulted in entodermization and exogastrulation. RUNNSTRÖM repeated the experiment of HERBST and envisaged the sea urchin egg as the site of two antagonistic gradients (1928, 1929, 1931, 1933), and this hypothesis was beautifully confirmed by the HÖRSTADIUS' comprehensive microoperative experiments (consult review by HÖRSTADIUS. 1939). Following this work, the analysis of such gradients on the biochemical level was attempted by numerous workers, some of whom have tested various vegetalizing agents, often using exogastrulation as the criterion of their efficacy.

However, as LINDAHL (1936) has pointed out, exogastrulation does not necessarily imply vegetalization. HÖRSTADIUS (1949), in fact, has cautioned against errors of this kind: "Often the great mistake has been made that exogastrulation is a sign of vegetalization . . . . We must also keep in mind that exogastrulation is due to lack of invagination and may appear without any increase of the endodermal region."

What, then, is the cause of this lack of invagination? The present paper will report a case of exogastrulation in *Pseudocentrotus depressus* which does not depend directly on any change of the developmental potencies, but rather indicates that the cause of the failure of invagination in this material lies in the lack of pseudopodia formation by the secondary mesenchyme cells.

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Material and Methods

The larvae of the sea urchin, *Pseudocentrotus depressus*, were exclusively used, because this form gives a higher percentage of exogastrulae with the Ca-low treatment used in this study than any of the other sea urchins available at Misaki. The optimum Ca concentration of the medium for exogastrula formation in *Pseudocentrotus* was found to be from 1/10 to 1/20 that of sea water. The solutions were prepared by mixing Ca-free artificial sea water* and the natural sea water in 9 : 1 and 19 : 1 ratios. For convenience, these media will be called "1/10 Ca" and "1/20 Ca", respectively, in the following description.

Two series of experiments were performed. In one the larvae were cultured continuously in the Ca-low sea water and in the other, they were temporarily exposed to the solution, and then returned to normal sea water. Parallel experiments were also run with LiCl and Mg-free or -low sea water for the purpose of comparison.

Results

I. Continuous Culture in Calcium-Low Sea Water

Larvae are transferred from normal sea water to 1/10 or 1/20 Ca at various stages, as indicated in Table 1, which shows the results of the experiment.

When the larvae are exposed to Ca-low sea water from the swimming blastula stage on, the highest percentage of individuals exogastrulate and the results are the most consistent. If the transference is made before hatching, the percentage of exogastrulae is rather low, especially in 1/10 Ca. If, however, the transference is deferred until the beginning of gastrular invagination, the figure again drops off. After completion of gastrulation, the larvae are scarcely affected by the treatment.

Comparison of normal and Ca-low larvae

Cases of transference made at the cleavage stage will be selected for the purpose of description.

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* NaCl 26.5 g., KCl 0.7 g., $MgSO_4 \cdot 7H_2O$ 11.9 g., $NaHCO_3$ 0.5 g. plus distilled water to 1000 cc.

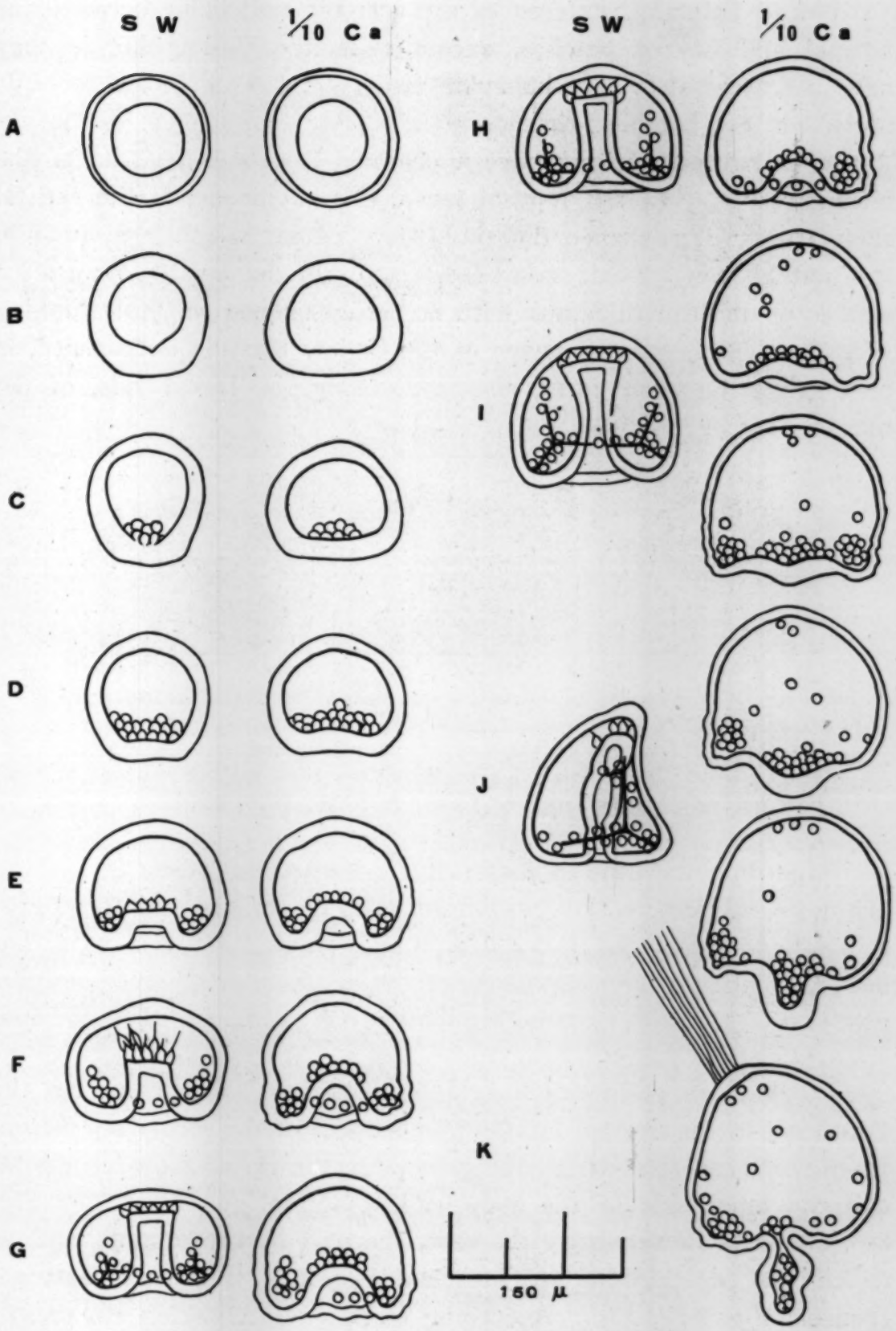


Fig. 1. Comparison of the developmental processes of normal and Ca-low larvae. × *ca.* 140.

Before hatching, differences are scarcely noticeable between the normal and Ca-low batches, except that the Ca-low larvae may have a somewhat thicker blastular wall (Fig. 1 *A*). No difference is noticed in the hatching time.

After hatching, a difference appears in the characteristics of the blastular wall. In the normal larva, this is thickest at the vegetal side with a wedge-shaped thinning at the center which is peculiar to the normal larva of this stage (Fig. 1 *B*). In the Ca-low larvae, the wall is of uniform thickness with no wedge-shaped vegetal thinning (Fig. 1 *B*). The overall shape of the Ca-low blastula is flattened, in contrast to the ovoid form, elongated along the larval axis, of the normal (Fig. 1 *B, C, D*).

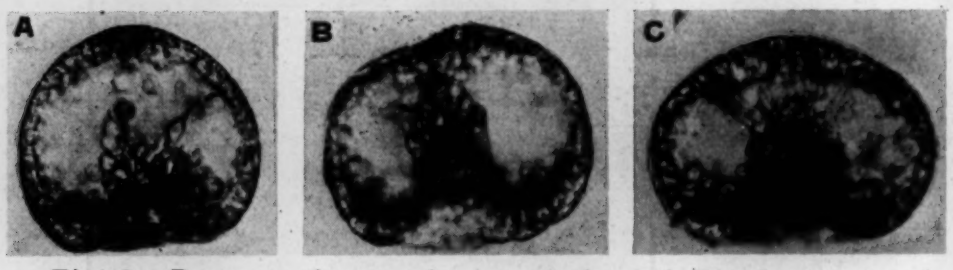


Fig. 2. Process of normal gastrulation of *Pseudocentrotus depressus*. × 195.
A: Pseudopodia of the secondary mesenchyme cells appear. *B–C*: Pseudopodial tips are attached to the body wall in the animal pole region. Note change of larval form along its axis.

Regarding the time of mesenchyme inward migration and of the beginning of invagination, no difference is detectable. At this stage, the wall of the Ca-low larva begins to wrinkle, and this wrinkling becomes particularly conspicuous at the position of the presumptive post-oral arms as development advances (Fig. 1 *E, F*; Fig. 3 *D*; Fig. 4 *B*).

When the archenteric invagination reaches half-way across the blastocoel in the normal larvae, pseudopodia of the secondary mesenchyme cells can be seen extending between the tip of the archenteron and the inner side of the body wall of animal pole (Fig. 1 *F;* Fig. 2 *B, C*). As is frequently the case, the part of the animal wall to which the pseudopodia are attached is pulled into the blastocoel (consult Fig. 3 *D′, E′*). According to DAN and OKAZAKI (in press), a function of these pseudopodia is to draw the archenteric tip toward the animal pole. Probably as a result of such contraction of the

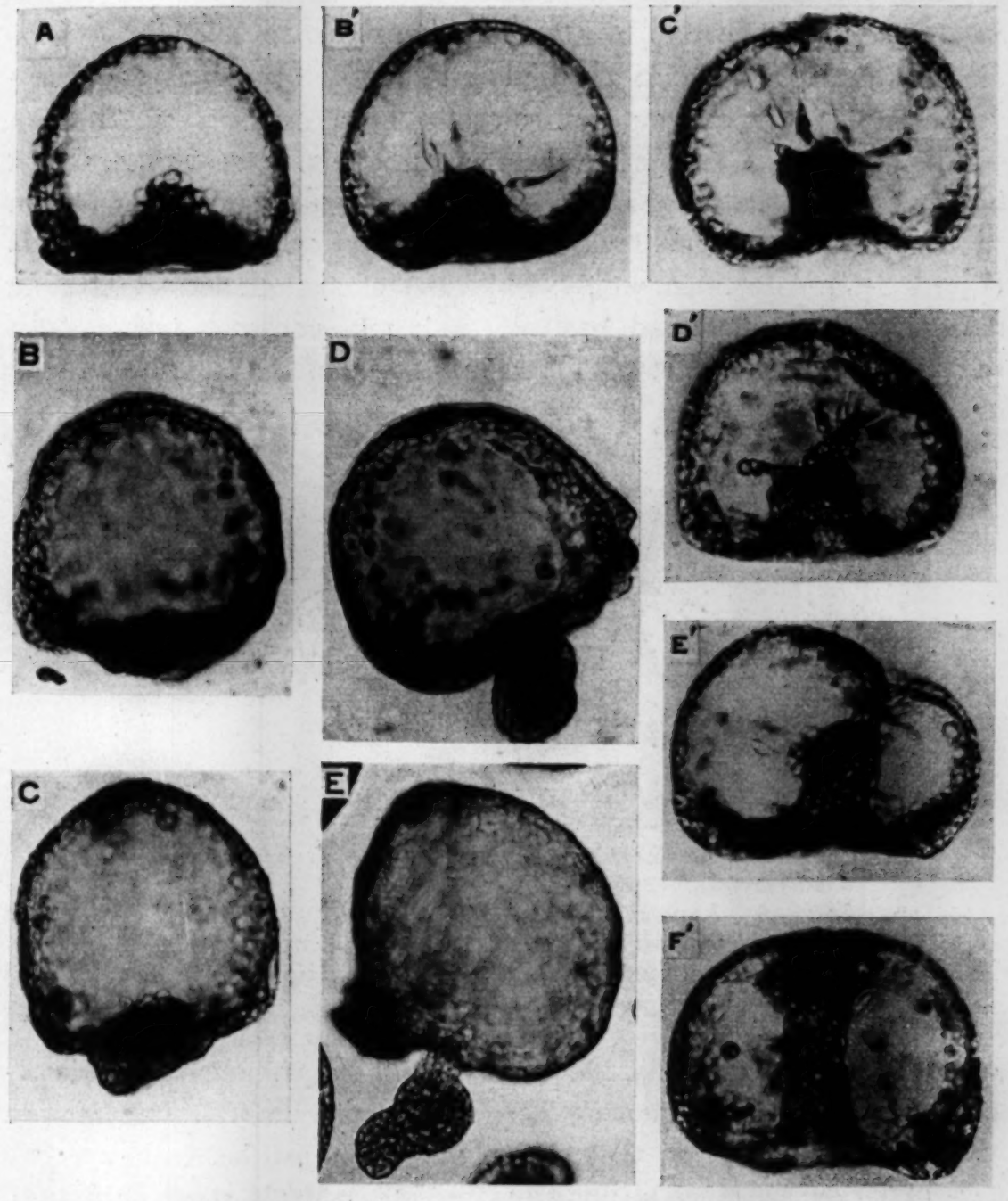


Fig. 3. Processes of exogastrulation in Ca-low media and resumption of invagination on being returned to sea water. ×195.

A–E: Process of exogastrulation in 1/10 Ca. *B'–F'*: Resumption of invagination when *A* is returned to sea water. *B'*: Appearance of pseudopodia. *C'*: Attachment of the pseudopodia to the body wall. *D'*, *E'*: Indentation of the gastrular wall as the result of pseudopodial contraction.

psedopodia, the previously elongated contour of the larvae suddenly becomes flattened (Fig. 1 *F*, *G*), and in very extreme cases, the larvae assume a shape like that of a mammalian red corpuscle.

On the other hand, in the Ca-low larvae, the mesenchymal pseudopodia fail to appear and the gastrular invagination stops in midcourse (Fig. 1 *F*, *G*). As development proceeds, these larvae, which have retained a flattened shape since the blastula stage, now begin to take on an elongated shape in the direction of the larval axis (Fig. 1 *E*–*G*). In other words, while the elongated normal blastula develops into a flattened gastrula with mesenchymal pseudopodia, the flattened Ca-low blastula grows into an elongated gastrula without

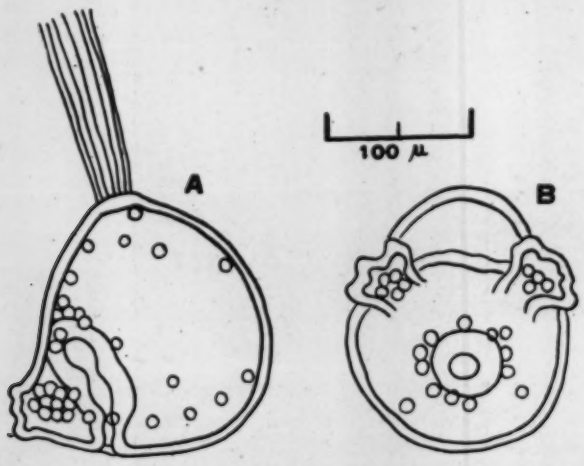


Fig. 4. Larva with invaginated archenteron in 1/20 Ca. × *ca.* 140.
A: Lateral view; Archenteron leans against the ventral wall, but constricts into three parts. *B*: Vegetal view; Wrinkling at the position of the presumptive post-oral arms.

mesenchymal pseudopodia. Moreover, while the volume of the blastocoel remains constant during normal gastrulation, this increases in the Ca-low gastrulae so that the volume difference between the two sets of larvae becomes striking by the late gastrula stage (Fig. 1 *G*), although both sets acquire equally long apical tufts (omitted from the figure).

As soon as gastrulation is complete, the blastocoelic size increases rather abruptly in the normal larvae (Fig. 1 *H*). Since this expansion occurs mainly along the larval axis, the normal larva once more becomes elongated and the archenteron becomes correspondingly longer. In Ca-low larvae, a similar enlargement of the blastocoel takes place

Table 1. Percentages of exogastrulae obtained by transferring *Pseudocentrotus* larvae to Ca-low medium at various stages

Concentration of Ca / Experimental series / Stage of transference to Ca-low		1/20				1/10			
		1	2	3	4	1	2	3	4
A		77	—	81	84	12	—	10	58
B		95	43	82	95	6	12	19	49
C		62	79	—	87	17	9	19	36
D		88	—	96	80	—	—	18	35
E		—	94	94	76	—	87	21	63
F		96	94	97	91	78	85	60	67
G		—	90	96	—	—	77	73	—
H		95	—	91	75	77	—	45	27
I		14	24	—	—	14	21	23	—
J		16	—	31	36	2	—	12	14
K		2	15	13	5	0	10	5	5
L		2	—	0	0	0	—	0	0

but with this, the archenteron gradually turns backward and exogastrulae result, following the course illustrated in Fig. 1 *H–K* and Fig. 3 *A–E*. The completely evaginated archenteron still possesses the faculty of self differentiation and develops a tripartite gut with the everted outside covered by ciliated epithelium (Fig. 1 *K*; Fig. 3 *E*). However, such an everted archenteron eventually falls away from the main body of the larva.

Archenterons of Ca-low gastrulae which are barely saved from becoming evaginated lean against the ventral wall and finally form small blind sacs constricted into three parts (Fig. 4 *A*). As development proceeds, the position of the blastopores of such larvae is gradually shifted from the central position toward the ventral side probably by a faster growth of the dorsal side of the larvae.

Transference of the larvae to the Ca-low medium at any stage before the initiation of invagination (Table 1, *D–H*) gives results which do not differ materially from the above. On the other hand, if it is done after the initiation of invagination (Table 1, *I, J, K*), the later the time of transference, the more larvae succeed in forming pseudopodia. Finally, if the transference is deferred until the pseudopodial connection is established (Table 1, *L*), the larvae are scarcely affected by the Ca-low treatment.

Comparison of Ca-low and LiCl exogastrulae

Exogastrulae of *Pseudocentrotus* resulting from Ca-low and LiCl treatment were closely compared, and the morphological differences noticed are summarized below:

1) In the Ca-low larvae, the archenteron invariably starts to invaginate at the beginning and only later gradually recedes as the blastocoelic volume increases, while in LiCl the corresponding part evaginates from the very beginning.

2) Entoderm is increased in amount by LiCl but not in Ca-low.

3) LiCl exogastrulae have no apical tuft, while Ca-low exogastrulae have a long apical tuft, as in the control.

4) The primary mesenchyme cells in LiCl larvae are shifted toward the animal pole in proportion to the effect of the agent, while they are found in the normal position in Ca-low larvae.

5) The blastocoel is larger in Ca-low but smaller in LiCl than the control.

6) The highest percentage of Ca-low exogastrulae is obtained by transference at the swimming blastula stage, at which the larvae have already become insensitive to LiCl.

Considering the above points, it will be clear that no such change in determination is brought about by Ca-low sea water as by LiCl.

Effect of Mg-low treatment

HERBST (1897) reported that entodermization takes place in the absence of magnesium ions, resulting in the formation of exogastrulae. It is true that in Mg-free or -low media, the larvae of *Pseudocentrotus* are more or less vegetalized (increased entoderm, inhibited apical tuft and displaced primary mesenchyme cells) and some larvae develop into exogastrulae. An attempt was therefore made to see what result would be obtained if the Mg-low condition were superimposed on the Ca-low condition. However, in 1/10 Ca–Mg sea water, no exogastrulae are formed. It was noticed that the larvae in 1/10 Ca–Mg have archenterons leaning against the ventral wall as was the case with Ca-low medium, although the blastocoels are smaller than those of the Ca-low larvae and the primary mesenchyme cells are shifted toward the animal pole to some extent.

II. RECOVERY AFTER RETURNING TO SEA WATER

Larvae kept in 1/20 Ca from the swimming blastula stage before inward migration of the primary mesenchyme cells were put back into sea water at various stages during the process of gastrulation, as is illustrated in Table 2.

If the larvae are returned to sea water early enough, while the entoderm still remains invaginated (Table 2, *A*–*C*), almost all the larvae are able to gastrulate successfully. Under this circumstance, the mesenchymal pseudopodia are formed and the invagination process follows the normal course, in spite of the rather large volume of the larvae (Fig. 3 *B′*–*F′*).

On the other hand, if the larvae are returned to sea water during the regression (Table 2, *D*) or after the evagination of the entoderm (Table 2, *E*, *F*), approximately 50% of the former and 90% of the latter develop into exo- or entoexo-gastrulae. This is probably because the pseudopodia are formed too late to take full part in the invaginating process. In the remaining 50% of the *D* lot and 10% of the

Table 2. Percentages of exogastrulae among *Pseudocentrotus* larvae which are cultured in 1/20 Ca from the swimming blastula stage to the various stages indicated

	Stage of return to sea water		Experimental series		
	S. W. control	1/20 Ca larva	1	2	3
A			0	—	0
B			—	2	2
C			—	4	5
D			49	—	51
E			84	87	—
F			94	—	—
Percentage of exogastrula when continuously cultured in 1/20 Ca			97	95	91

E and *F* lot, the archenterons are frequently abnormal in many respects, despite the fact that they remain inside the blastocoel. In such larvae, the tips of the mesenchymal pseudopodia fail to reach the animal pole and attach at random to any part of the larval wall (Fig. 5 *A*). Consequently, the archenteric tips are drawn to these unsuitable places, a result which, in turn, prevents the union between the archenteron and the stomodaeal invagination although the latter develops independently at the normal position (Fig. 5 *B*, *C*). Finally, it must be emphasized that all these gastrulae produced by the Ca-low treatment, whether their archenterons evert or not, develop, if

they are returned to sea water, into plutei which are quite normal except for their abnormal archenterons and unpaired coeloms (Fig. 5 *B*, *C*).

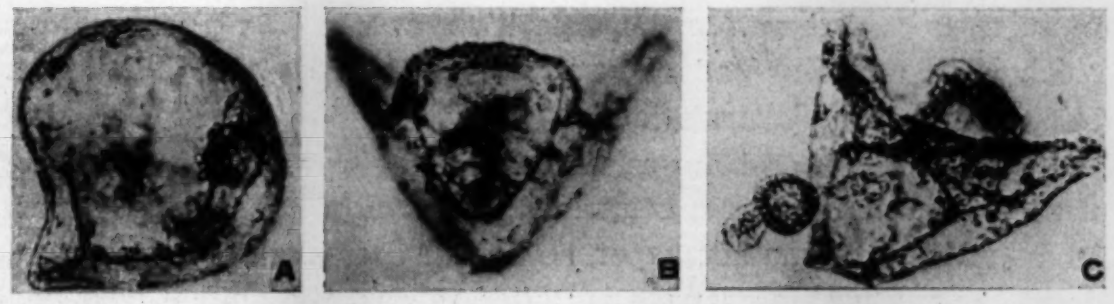


Fig. 5. Larvae obtained by temporary Ca-low treatment. × 120.
A: Archenteron is inclined toward the dorsal wall. *B*: Pluteus derived from *A*. Note an unpaired coelom intervening between the tip of the archenteron and the stomodaeal invagination. *C*: Pluteus derived from 1/10 Ca exogastrula. Stomodaeal invagination develops in the normal position independently of position of archenteric tip (as also in *B*).

Discussion

Following the discovery by HERBST of LiCl as an exogastrula-forming agent, numerous authors added more agents to make up a varied list; *e.g.* sodium butyrate (HERBST, 1896), lack of magnesium (HERBST, 1897), NaCl, $HgCl_2$, $CuSO_4$, KCN, etc. and their salt mixtures and hypotonic sea water (MACARTHUR, 1924; WATERMAN, 1932, 1934), neutral red (MACARTHUR, 1924; CHILD, 1936), Co (RUNNSTRÖM, 1928), auxin, glycogen and $KClO_3$ (MOTOMURA, 1934), methylene blue and tobacco smoke (CHILD, 1936), NaCNS (RULON, 1941), sodium azide (CHILD, 1948, 1953), changes in temperature (DRIESCH, 1895; WATERMAN, 1934), crowding (MACARTHUR, 1924; CHILD, 1936), X-rays (WATERMAN, 1934), etc. However, the above list includes some agents of which the vegetalizing power is dubious or which have even proved to be animalizing. In spite of this, the notable success of the gradient theory, in which the effect of lithium ions is interpreted as bringing about vegetalization and eventually the formation of exogastrulae, seems to have so strongly impressed investigators that some of them have been misled to think that exogastrulation is invariably a result of vegetalization whether it is caused by LiCl or by other agents.

The Ca-low exogastrula described in this paper is a concrete example which indicates that exogastrulation can also be brought about

by other means besides vegetalization, because Ca-low exogastrulae do not show any of the characteristics by which lithium larvae are judged to be vegetalized and Ca-low larvae are saved from exogastrulating if they are returned to sea water before the archenterons begin to regress. Furthermore, superposition of the Mg-low condition, which is vegetalizing and exogastrula forming, on the Ca-low condition prevents exogastrulation altogether.

Obviously, the problem arises as to what was the direct impediment in the morphogenic course of gastrulation in the present case. As is reported in another paper (DAN and OKAZAKI), the archenteric tip of the sea urchin gastrula is pulled toward the animal pole body wall by the pseudopodia of the secondary mesenchyme cells, and if the pseudopodial connection is artificially severed, an exogastrula results. In the present material, the following two facts were constantly observed; 1) With continuous culture in Ca-low sea water begun in the blastula stage, the pseudopodia formation is completely supressed and an exogastrula results. 2) On returning Ca-low larvae to sea water, successful invagination is preceded by the devolopment of the pseudopodia. The consistency of this relation provides strong evidence that the direct cause of Ca-low exogastrulation is to be found in the lack of pseudopodia formation.

However, in Ca-low sea water before evagination begins, the entoderm first invaginates as far as the center of the blastocoel independently of pseudopodia formation. No satisfactory explanation has yet been found with respect to this point.

The author wishes to express her sincere thanks to Professor K. DAN for his valuable suggestion and to the director and the staff of the Misaki Marine Biological Station for use of the Station's research facilities. Her thanks are also due to Dr. J. C. DAN for her kind advice in the preparation of the manuscript.

Summary

1) Exogastrulae have been secured in high percentage by exposing the larvae of *Pseudocentrotus depressus* to a Ca-low medium (1/10 to 1/20 normal calcium level of sea water).

2) In the process of exogastrulation in the Ca-low medium, the archenteron invaginates 1/2 to 1/3 the diameter of the blastocoel at the start, and then gradually retreats and finally turns completely out.

3) Such exogastrulation is correlated with the absence of the pseudopodia of the secondary mesenchyme cells.

4) When *Pseudocentrotus* larvae cultured in Ca-low medium are returned to sea water before regression of the archenteron, they succeed in invaginating. This is preceded by pseudopodia formation.

5) In contrast to LiCl-treated larvae, Ca-low larvae do not show the evidences of change in determination associated with vegetalization.

6) *Pseudocentrotus* larvae do not develop into exogastrulae following treatment with Ca-Mg-low sea water.

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STUDIES ON THE "DOUBLE NUCLEUS" IN THE EARLY DEVELOPMENTAL STAGE OF *BUFO VULGARIS*

II. ON THE FREQUENCY OF DOUBLE NUCLEUS UNDER VARIOUS EXPERIMENTAL CONDITIONS, WITH SPECIAL REFERENCES TO THE OXYGEN CONCENTRATIONS OF CULTURE MEDIA

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INTRODUCTION

In the first report of this series of studies (SIRAKAMI, 1956), the author described the amitosis-like "double nucleus," which was found frequently in the ectodermal region of *Bufo vulgaris* during the normal course of gastrulation. Although its possible participation in the process of cell multiplication was discussed from a quantitative cyto-embryological standpoint, its true nature and significance were left untouched. In 1931 Stough proposed the idea that the amitosis is a modification of the normal mitosis which is brought about "as a concession to the speed required in a rapidly growing chick embryo." But he did not explain how and why the normal mitosis was modified.

In this paper the effect of dissolved oxygen upon the frequency of double nucleus was studied. It was found that the double nucleus disappeared almost completely when the embryo was cultured in pure oxygen at high temperature and that, on the contrary, the double nucleus was formed abundantly under deficient oxygen supply.

In the next place, the effects of various chemicals, including some metabolic inhibitors, upon the double nucleus and upon the normal mitosis were investigated under suitable oxygen concentrations of culture media.

The Effect of Oxygen Concentrations on the Double Nucleus

Methods

A simple method was devised to control the oxygen concentration of culture media. Wide-mouthed bottles containing about 500 cc. of oxygen, nitrogen, and their various mixtures with air were prepared. Into each bottle, ten embryos were quickly introduced with small quantities of culture media and the bottles were then installed in thermostats adjusted to suitable temperatures. In practice, some 30 bottles with various gas mixtures and at various temperatures were used at a time. When the embryos reached a desired developmental stage between the blastula and the crescent blastopore stage, the bottle was opened and the embryos were fixed for further microscopical investigations. By means of Winkler's method it was found that one can control the oxygen concentration of the medium, ranging from 5 to 450% of the normal level. Here the normal level implies the concentration of 0.68 cc. oxygen NTP in 100 cc. of medium, corresponding to that of tap water saturated with air at 18° C., which hereafter will be expressed as DO : 100 in this paper.

The culture medium was continuously in contact with about 450 cc. of gaseous phase. A shifting in the equilibrium point due to the respiration of embryos might be neglected, since ten embryos consume less than 1/100 cc. of oxygen in 24 hours at 20° C.

Embryos were fixed by Zenker's fluid and were cut into sections 10 micra in thickness by paraffine method and stained by the Feulgen's nucleal method. Frequencies of the double nucleus and the mitosis were determined in the ectodermal region. Staining by water-blue picric acid was found generally inadequate for our present purpose, because it did not give clear picture of the mitotic chromosomes. In the course of studies, it was found necessary to draw volume distribution curves of the single nucleus by the method described in the preceding paper (Sirakami, 1956) in order to get some idea about activity or splitting of the double nucleus.

Results and their interpretation

In the normal embryo, cultured in air at the room temperature (12°–16° C.), the frequency of double nuclei is more than 15% and that of normal mitoses is about 5% at the crescent blastopore stage

(st. 13 by BABA-OKADA, 1932). If we change oxygen conditions and temperature of the culture medium, the result widely deviates from the above mentioned normal levels. First we consider the results at high temperature (28° C.). Data presented in Table 1 A and 1 B show that in the same ectodermal region the double nuclei are very rare if the embryos are cultured in pure oxygen, and abundant if cultured in air at the same temperature. On the other hand, the mitotic figures behave in a precisely reversed manner, that is, they are scarcely observed if the embryos are cultured in air. Because in both cases only the amount of dissolved oxygen differs (250 : 70, in our DO units), these results seem to suggest that the double nucleus is replaced by the normal mitosis when sufficient oxygen is available.

However, experiments at the room temperature (12°–16° C.) give different results: The percentage of double nucleus is considerably high even in pure oxygen (Table 1 D). With decreasing oxygen concentration the value remains unchanged or even decreases gradually (Table 1 E, 1 F).

At both temperatures the frequency of the double nucleus sinks to the lowest level, if air is driven away from the culture media by bubbling hydrogen or nitrogen, keeping DO less than 10. Especially in the series of high temperature double nuclei disappear almost completely (Table 1 C).

How can we interpret all these results harmoniously? Here we need information about the kinematic aspect of the situation. So we turn to the distribution curves of the volume of single nuclei at the crescent blastopore stage under various experimental conditions. As shown in the preceding report (SIRAKAMI 1956) and also in Table 2, the volume of single nuclei displays typical unimodal distribution during the normal course of gastrulation (DO : 100, room temperature). When the crescent blastopore stage is attained under high oxygen concentration at room temperature, the double nucleus does not disappear and is sometimes even increased in frequency (Table 1 D), whereas the volume distribution curves of single nuclei still retain their unimodal V-shape (Table 2 and Fig. 1). On the contrary, if the dissolved oxygen is decreased and the frequency of double nucleus only slightly decreased, the curves take bimodal W-shape (Table 2, Fig. 1).

Table 1. Frequency of the mitosis and the double nucleus in the ectodermal region at the crescent blastopore stage of *Bufo vulgaris*, cultured with various gas phases at various temperatures

	Conditions	Mitotic index %	Double nucleus %	Cells counted	Shape of volume distribution curves
A	Gaseous phase: Oxygen Temperature: 28° C. Dissolved oxygen: DO: 250–300	8.0 3.0 3.3 4.8 3.0 2.8 9.3 7.1 7.0 5.0	0 2 0 0.5 0.6 0.5 0 0 0 0	813 609 1,109 778 885 726 915 1,030 605 518	V
B	Gaseous phase: Air Temperature: 28° C. Dissolved oxygen: DO: 50–70	0.3 0.9 4.0 2.9 1.6 2.2 1.2	6.5 2.4 15 10 5 20 18	364 396 694 972 560 406 1,000	W
C	Gaseous phase: Hydrogen Temperature: 28° C. Dissolved oxygen: DO: 5–10	2.0 5.0 4.8 5.3 3.0 4.0 6.2 4.0 4.4 6.7 6.2	0 2 3 0 2 3 0 0 3 2 2	1,000 808 706 855 825 1,000 518 516 545 656 471	W
D	Gaseous phase: Oxygen Temperature: about 12° C. Dissolved oxygen: DO: 400–450	7.4 11.7 8.0 8.6 9.0 9.2 8.0 8.5	15 20 15 10 13 8 15 10	475 448 463 473 459 458 486 461	V
E	Gaseous phase: Air Temperature: about 14° C. Dissolved oxygen: DO: about 100 (Control)	3.5 4.1 3.0 2.8 2.5 3.7 4.7 3.8	11 10 10 15 11 9 18 15	1,250 1,002 741 1,000 1,000 1,000 732 830	V
F	Gaseous phase: Hydrogen and Nitrogen Temperature: about 14° C. Dissolved oxygen: DO: 10–20	6.6 6.4 3.9 3.4 4.9 3.2	15 10 15 10 10 10	1,000 1,000 1,000 1,076 1,000 1,000	W

Table 2. Percentage volume distribution of single nucleus at the crescent blastopore stage under various oxygen conditions

Gaseous phase, Temperature, Dissolved oxygen	Classes of the mean radius (1 unit : 0.67 μ)											Shape of curves
	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5 10.0	
O_2 14° C. (DO : 400–450)				1	5	19	26	**33**	14	1		V
						4	15	**38**	28	13	2	V
					1	2	10	24	**33**	24	5	V
O_2 28° C. (DO : 300–400)				9	22	**35**	21	11	2			V
	1	3	6	14	**27**	**27**	17	5				V
				3	18	**35**	31	12	1			V
Air 14° C. (DO : 100) Control				4	22	**38**	28	7				V
			3	9	13	**36**	20	16	3			V
				3	12	**35**	28	17	5			V
		2	5	18	**37**	24	13	2				V
			5	13	**32**	25	18	7				V
			1	14	**33**	29	16	5	2			V
Air 28° C. (DO : 50–70)	5	11	15	16	18	**22**	7	1				U
	9	10	11	9	**20**	17	10	4				W
H_2 28° C. (DO : 5–20)	6	10	**20**	**20**	16	14	14					U
				3	18	**23**	18	20	11	6	2	W
				2	2	8	17	**25**	23	16	7	U

Dissolved oxygen concentrations are expressed in DO units (DO : 100 corresponds 0.68 cc. of oxygen NTP in 100 cc. of culture medium). For identification of the shape of curves, see in the text.

The same relations exist also in the case of high temperature (Table 2). In pure oxygen the curve assumes V-shape, while in air U- or W-shaped curves are obtained. Thus a deficiency in oxygen seems to cause the transformation of the volume distribution curve from V-shape into W-shape under these experimental conditions.

In short, various oxygen concentrations of culture media modify the frequency of double nucleus and at the same time the shape of volume distribution curves of single nucleus. When there is only a small number of double nuclei, we find two types of volume distribution curves of single nucleus (Table 2): the V-shaped curve, as is observed under high oxygen concentration at high temperature; and the W-shaped curve, as is observed under deficient oxygen supply. The percentages of the most frequent class are expressed in gothic letters in Table 2. It might be generally stated that if this value is over 30%, the distribution curve assumes a V-shape and if the value

is less than 25% the curve is W-shaped. Between these values the curve may take a W-shape or an obtuse U-shape.

It is well known that gastrulae of *Bufo vulgaris* can continue their development under considerably low oxygen concentration (for instance, DO : 10, *i.e.* 1/10 of the normal level) at least to the tail bud stage (st. 22). The maximal developmental velocity at the same room temperature is observed at a slightly lower oxygen concentration (DO : 50). It is reported earlier by the author (SIRAKAMI, 1955) that at the tail bud stage cell number in ectodermal region attained, even under deficient oxygen concentrations, the same level of magnitude as that of the normal embryo (about 40,000 to 50,000 in the neural tube).

This might suggest that the process of cell multiplication proceeds regularly within rather wide range of oxygen concentrations, which on the other hand actually affect the shape of distribution curves of the single nucleus as well as the frequency of double nucleus. In other words, cells in the ectodermal region must be assumed to multiply at a high rate comparable to the normal one during the course of gastrulation even under deficient oxygen supply.*

Under higher oxygen concentrations and at high temperature, where double nucleus is only rarely found and the volume distribution curves of single nucleus are V-shaped, we assume that the production as well as the fission of double nucleus is really inhibited. But, with deficient oxygen supply, the shape of volume distribution curves changes from ordinary V-shape into W-shape. The rare occurrence of double nucleus in such cases might be explained that the double nucleus is really being formed but is splitting into two daughter single nuclei so rapidly that it has little chance to be detected in a state of typical double nucleus. This assumption appears to be supported by the fact that the ratio of diametres, corresponding to each peak of the volume distribution curve, is approximately $\sqrt[3]{2} : 1$ (comp. Table 2 and Fig. 1). The one peak perhaps corresponds to the diameter of partners of double nucleus having just divided, because their volumes are about half as large as the ordinary single nucleus (SIRAKAMI, 1956).

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* This statement is also supported by our recent direct observations of the actual course of multiplication of isolated blastomeres cultured under various oxygen concentrations. Deficient oxygen (DO : 20) does not affect the multiplication rate of the isolated blastomeres.

Thus one might assume that in pure oxygen at room temperature the double nucleus *accumulates* because its fission with cytoplasmic division is inhibited and that, under low oxygen concentration, the fission is so much accelerated that one can observe only a small fraction of double nuclei in their typical paired forms in a microscopical section. At high temperature, also the production of the double nucleus may be inhibited by excessive oxygen. Cells are then supposed to multiply mainly by normal mitosis.

Since it is known that cells in the gastrula can multiply very rapidly within a wide range of the oxygen concentrations, it is interesting to note that the mode of cell multiplication in these periods seems to be doubly assured by normal mitosis and by the formation of double nucleus.

Of course the occurrence as well as the behaviour of double nuclei under various oxygen concentrations, as observed in the present experiment, are by no means general phenomena through the whole developmental stages of *Bufo vulgaris*; their appearance is confined to a particular phase of development, *i.e.* the gastrulation. This phase specificity might be correlated to metabolic processes peculiar to this stage.

Frequency of the Double Nucleus under High Oxygen Concentrations as Affected by Some Chemicals

In order to get some idea about the mutual relationship between double nucleus and mitosis, effects of some metabolic inhibitors upon the frequency of their occurrence were studied. The fact that we are now able to control the frequency of double nucleus by changing the concentration of dissolved oxygen facilitated the present series of experiments.

As stated in the preceding section, if excessive oxygen is applied at high temperature (28° C., *i.e.* about 4° C. below the lethal temperature), both the production and the fission of double nucleus seem to be suppressed. Cells are then supposed to multiply exclusively by means of typical mitosis, becuase the development and cell multiplication go on apparently normally.

However the following experiments show that some metabolic inhibitors cause re-appearance of the double nucleus when applied under such conditions.

Methods

The following chemicals were tested for their effect on the appearance of double nucleus: potassium cyanide, 2-4-dinitrophenol, lactic acid, potassium thiocyanate, mono-iodo acetic acid, sodium azide, sodium fluoride, trypaflavine etc. Series of solutions of graded concentrations were prepared for chemicals to be tested. Blastulae were put into solutions and enclosed in bottles filled with pure oxygen. When the embryos developed to the crescent blastopore stage, the bottle was opened, the embryos were then fixed in ZENKER's fluid and studied as above mentioned.

The term "critical concentration" was used in this paper to denote the maximal concentration of the tested chemicals, in which the embryos survive and continue to gastrulate at least to the crescent blastopore stage. In some cases the development was stopped at this stage at the critical concentration.

Results

The effects of each chemical were studied in respect to the frequency of double nucleus, the mitotic index, and the shape of volume distribution curves of the single nucleus. The results are summarized in Table 5 (p. 53).

The results will be presented separately for each chemicals.

(*a*) *Potassium cyanide*

KCN solutions of different concentrations were prepared and were adjusted by N/10 HCl to pH 7. Solutions were enclosed in bottles in order to prevent the evaporation of HCN, but their actual concentrations were hard to determine.

As is well known, embryos of *Bufo vulgaris* can resist the action of KCN as well as the shortage of oxygen. They gastrulate even in the presence of 30 mg. % of KCN. Such a resistance may be largely due to low permeability of the vitelline membrane and the surface coat. As the embryo develops, the susceptibility increases and the minimal lethal concentration for the tail bud stage drops to 1/8 mg. %. Therefore the embryo must be cultured in KCN of 1/8 mg. % to allow the development beyond the gastrula stage.

In embryos cultured in KCN solution with concentrations varying from 1/8 to 30 mg. %, a strong suppression of the mitotic figures was always observed (Table 3). The double nucleus was abundant, and the volume distribution curve of single nucleus changed into U- or W-shape (Table 3 A and Fig. 1). It might be stated that KCN

Table 3. Modification of percentage distribution of nuclear volumes by various chemicals with sufficient (A) and deficient (B) oxygen supplies

	Chemicals	Classes of mean radius (1 unit: 0.67 μ)												Shape of curves
		4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0	
A DO: 300–400 28° C.	KCN 2.5 mg. %				1	0	2	14	15	19	**23**	17	8	U
	KCN 5 mg. %		1	3	9	20	20	**23**	14	10				W
	KCN 5 mg. %			1	6	16	19	**21**	17	9	9	2		U
B DO: 50–20 28° C.	DNP 1/8 mg. %		2	1	5	10	17	**29**	20	10	4	2		V
	DNP 1/8 mg. %			2	6	17	35	**36**	3	1				V
	NaF 10 mg. %	2	6	17	20	**32**	16	6	1					V
	NaF 10 mg. %			2	7	15	16	**21**	**21**	10	7	1		U
	MJE 5 mg. %		1	5	16	26	**36**	12	3					V
	MJE 10 mg. %		9	20	**30**	25	13	3						V
	ATP 20 mg. %		3	7	26	**30**	24	8	2					V
	ATP 20 mg. %		1	7	17	**29**	27	17	1	1				V
	ATP 20 mg. %		3	12	24	**39**	19	3						V
	LiCl 1/10 M			2	22	**37**	25	12	2					V
	LiCl 1/10 M				5	8	22	**32**	22	10	1			V

In the normal embryo sufficient oxygen causes V-shaped, dificient oxygen U- or W-shaped distribution of volumes of the single nucleus. The shape of frequency distribution curves can be roughly guessed by the percentage value of maximal frequency (expressed in gothic letters). Further explanation is in the text.

produces, even in the presence of excessive oxygen, a condition similar to those under deficient oxygen supply at the crescent blastopore stage.

(*b*) *2, 4-dinitrophenol* (*DNP*)

The action of DNP was in a distinct contrast to that of KCN, being more toxic for the earlier stages. A 1/3 mg. % solution of neutralized DNP caused severe general cytolysis and spherical condensation of nuclei in the ectodermal region of the late blastula and marked inhibition of the development. In 1/6 to 1/8 mg. % solution the blastula scarcely attained the crescent blastopore stage. Microscopical observations revealed relatively high frequency of double nucleus with considerable numbers of mitotic figure (Table 4 p. 51). Sometimes all the resting nuclei were dilated, and some degenerating nuclei were seen as FEULGEN-positive droplets scattering within the cytoplasm. Because the mitotic figure is always observed in considerable number and in apparently unaffected condition, the above mentioned degenerating nuclei may not be ascribable to the mitotic chromosome.

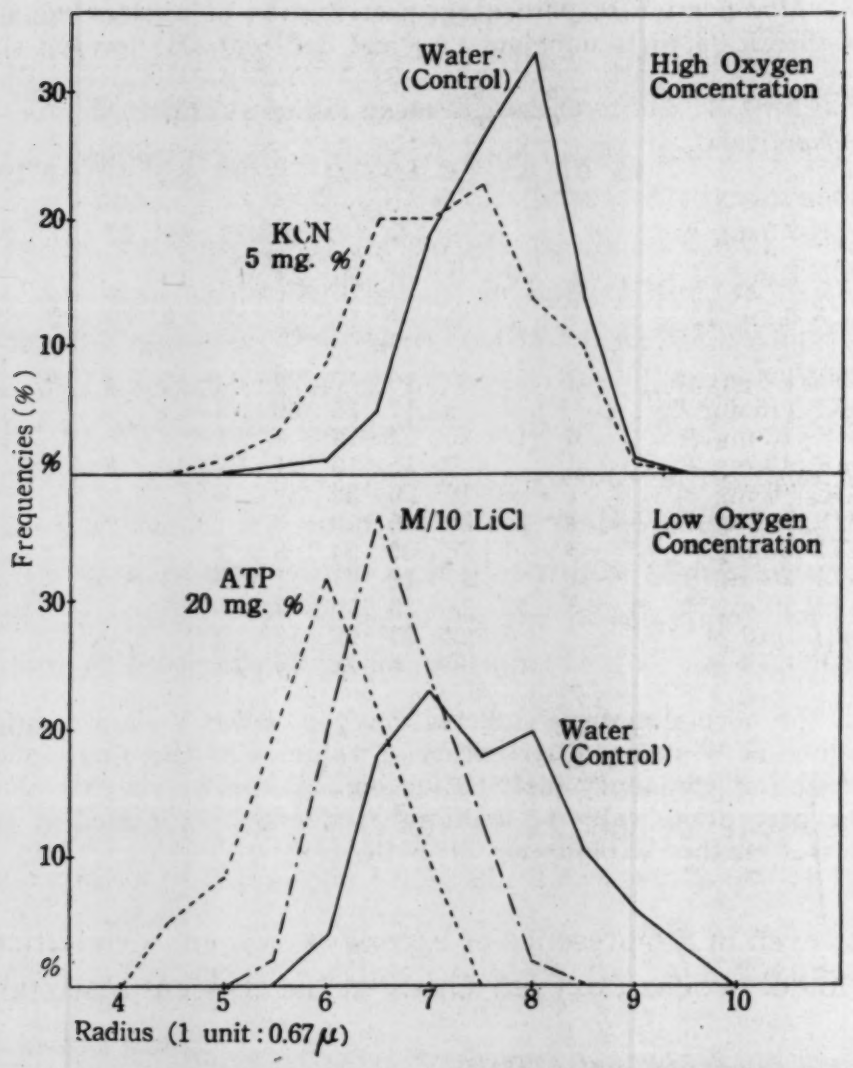


Fig. 1. Transformation of the shape of percentage volume distribution curves of single nucleus under various oxygen concentrations and by some chemicals.

The volume distribution curve of the single nucleus was always V-shaped (Table 3 B), suggesting that the fission of double nucleus was completely arrested.

It was noticed that the range of DNP concentration for its specific action was considerably narrow; 1/3 mg. % was already lethal for the blastula, while in solutions less than 1/10 mg. % the development went on normally without any observable cytological change.

(*c*) *Lactic acid*

The critical concentration of lactic acid for the blastula ranged from M/400 (pH 3.0) to M/800 (pH 3.5), in which the embryos developed to the crescent blastopore stage. Microscopical observations

indicated that the external layer of ectoderm was heavily damaged, while the internal parts remained intact. The chromosomes were absent and some ectodermal and mesodermal cells contained caryorhectic drops of chromatin corresponding perhaps to the cells which were in mitosis. Most of the resting nuclei in relatively intact internal parts were somewhat dilated, while the concurrent double nuclei, though very small in number, seemed to be exempted from the general destruction. Thus the mitotic chromosomes appeared most sensitive, the resting nucleus less sensitive, and the double nucleus most resistant to the action of lactic acid.

Because it is known that lactic acid accumulates during the gastrulation (G. TEN CATE 1953), the cell multiplication by means of double nucleus might be here more advantageous than the normal mitosis, the former being supposed to stand much higher concentrations of lactic acid than the latter.

(*d*) *Other chemicals*

The results obtained by other chemicals (potassium thiocyanate, mono-iodo acetic acid (MJE), sodium azide, sodium fluoride, trypaflavine, etc.) are summarized in Tables 3, 4 and 5.

The effects of poisons upon the embryonic body as a whole are surely manifold and complicated, but if we confine our attention to the frequency of double nucleus and mitosis, the results are rather simple and can be characterized as follows. Almost all the chemicals employed in this study induced re-appearance of double nucleus in the critical concentrations even under high oxygen concentration, which by itself alone inhibits production and fission of double nucleus. On the other hand, the distribution curves of volume of single nucleus were found to be always V-shaped. These tendencies might suggest that chemicals in their slightly injurious concentrations cause the appearance of double nucleus, whereas they do not, except for KCN and NaF, promote its splitting.

Potassium thiocyanate. The critical concentration for arresting the gastrulation was about 1/10 M. Abundant double nuclei were observed in this concentration, with very rare mitoses. The mitotic figures, if present, were quite intact. The injury might be stated to be mainly cytoplasmic, because the cytoplasm showed sometimes

strange aspects of polarization, that is, pigment granules flocked to the outer portion of each ectodermal cell.

Mono-iodo-acetic acid. The critical concentration was about 1/400 M. At this concentration the effect might be confined to the external layer of the embryo, where cells were very large, containg pale and dilated nuclei. As for the nuclear structures, interior parts appeared quite normal.

Sodium azide. In 1/10,000 M solution the frequencies of both double nuclei and mitoses were lower than the normal levels. Sometimes almost all the cells showed constriction of nuclei and general vacuolization of cytoplasm. Unlike KCN, the distribution curves of single nucleus were always V-shaped, suggesting that the fission of double nucleus was not promoted.

Trypaflavine. The effect of this chemical was mainly caryoclastic at the critical concentration (1 mg. %): spherical condensation of the mitotic chromosome was observed with considerable number of intact double nuclei. The distribution of volume of single nucleus was unimodal and there can be expected no cell multiplication at higher concentrations.

Effect of Chemicals under Low Oxygen Concentrations

When the temperature of culture medium is elevated to 28° C. in the air (DO : less than 70) or when the medium is enclosed in bottles with pure hydrogen or nitrogen, the concentration of dissolved oxygen diminishes markedly (DO : less than 20). The early blastula can gastrulate under such quasi-anaerobic conditions and produces a large number of double nuclei in the ectodermal region. As shown above (Table 1), the volume distribution curves of single nucleus take W-shapes, suggesting that the double nucleus is then actively in division. If various chemicals are applied under such condition of oxygen tension and temperature, we may be able to study their direct effects upon the double nucleus. As was already pointed out in the preceding section, many chemicals cause the production of double nucleus without changing the form of distribution curves of single nucleus under high oxygen tensions. The experiment in this section will answer the question whether they affect the splitting of double nucleus under deficient oxygen supply.

(*a*) *Lithium chloride* (*LiCl*)

It is well known that LiCl causes serious developmental defects in the neural and notochordal regions when applied to the early developmental stages of different chordates. Unlike other general protoplasmic poisons, LiCl is most toxic when applied at a certain stage of development, namely at the late blastula in *Bufo vulgaris*. It is rather surprising that almost no cytological studies have been made concerning the direct action of LiCl upon amphibian embryo, especially at the sensitive period.

The embryos with the intact vitelline membrane were allowed to develop in M/5 and M/10 LiCl from the blastula to the crescent blastopore stage. These concentrations were somewhat too strong



Fig. 2. Coagulation figures of the resting nucleus caused by M/5 LiCl at 28° C. in air. Note that a cell is in mitosis. Fixing by ZENKER's fluid and staining by HEIDENHAIN's hematoxyline. (×1,200)

for embryos to continue their development. In M/20 solution embryos can develop beyond the tail bud stage, though sometimes with slight microcephalic appearances.

The most striking feature of the cytological action of LiCl was the disappearance of double nucleus in the normal and lower oxygen concentrations.

The volume distribution curves of single nucleus were perfectly V-shaped (Table 3), suggesting that both the production and fission of double nucleus were really suppressed. On the other hand, the mitotic index was even somewhat elevated (Table 4). Probably the typical mitosis is going on under such conditions, maintaining the necessary process of cell multiplication by this mode of cell division.

In 1/5 M solution, all the resting nuclei contained coagulated mass



Fig. 3. Marginal zone from an early gastrula treated by ATP (20 mg. %). Almost all the cells are in mitosis. Fixing by BOUIN's fluid and staining by water-blue picric acid. (×650)

of chromatin in the centre, while the peripheral zone was transparent with intact nuclear membrane (Fig. 2). Sometimes coagulated masses assumed the shape of a dumb-bell. These figures might correspond to the double nuclei at the moment of production.

It is very interesting to note that quite intact mitotic figures were sometimes observed even in such a strong solution (Fig. 2).

(*b*) *Adenosine-triphosphate* (*ATP*)

During the earlier phases of our investigation, no distinct effect of

Table 4. Effect of chemicals upon the frequency of the mitosis and the double nucleus at the crescent blastopore stage in high and low oxygen concentrations

Conditions	Chemicals	Concentration	Mitotic index %	Double nucleus %	Cells counted
	KCN	30 mg. %	1.7	30	692
		〃	2.3	40	596
		5 mg. %	1.7	30	295
		〃	0.8	30	496
		〃	0.6	30	497
		2.5 mg. %	1.5	30	542
		〃	1.7	30	714
		〃	1.1	30	603
Gaseous phase: Oxygen	DNP	1/8 mg. %	6.5	10	570
Temperature: 28° C.	NaF	10 mg. %	5	10	500
Dissolved oxygen:		〃	4	10	500
DO: 250–300	KCNS	100 mg. %	2	5	500
		〃	3	5	500
		〃	2	5	500
		〃	3	5	500
	NaN_3	10^{-4} M	3	4	500
		〃	5	3	500
		2×10^{-4} M	3	10	500
		〃	3	13	500
	LiCl	1/10 M	3	0	500
		〃	4	0	500
		〃	5.3	0	546
		〃	9.1	0	733
Gaseous phase:	MJE*	10 mg. %	2	0	500
Air or nitrogen		〃	1	1	500
Temperature: 28° C.	ATP	20 mg. %	5.3	10	1045
Dissolved oxygen:		〃	7.8	10	1020
DO: 10–50		〃	12.7	10	976
		〃	8.0	10	926
		〃	10.7	10	542
		〃	5.7	10	473

* Mono-iodo-acetic acid.

ATP upon the embryos was noticed; the frequency of double nucleus and mitosis showed no marked change from their normal levels. However, exceptionally abundant mitotic figures were sometimes found in the ectodermal regions. Further the volume distribution curves of single nucleus changed from W-shape to V-shape (Fig. 3 and Table 3). An inhibition of the fission of double nucleus by ATP (20 mg. %) under low oxygen concentrations is thus suggested.

(*c*) *Other chemicals*

The results are summarized in Tables 3, 4 and 5.

It can be stated that the caryo-toxic properties of chemicals were somewhat intensified under low oxygen tensions, but their general effects did not essentially differ from those under high oxygen concentrations. The double nucleus was always abundant and the volume distribution curves of single nucleus generally remained V-shaped (Table 3). The results indicate that the chemicals employed in the preceding section actually inhibit the fission of double nucleus even in low oxygen concentrations, which are supposed otherwise very favourable for the splitting of double nucleus in the normal embryos.

Sodium fluoride forms a single exception to the above statement, the volume distribution curves of single nucleus showing U- or W-shape at the critical concentration (Table 3). This exceptional situation might be connected with the fact that the injury was confined to the outer layer (as with mono-iodo-acetic acid, p. 48). However, delayed separation of chromosomes and other pathological changes were observed in deeper layers of the ectoderm as well.

Mono-iodo-acetic acid. The critical concentration (5 mg. %) was somewhat lower than that in higher oxygen concentrations. The morphological effect of this chemical was similar to that of sodium fluoride. The outermost layer of cells in the ectoderm were very large with dilated nuclei. The problem, whether these injuries are based upon the arrest of the cell division or upon the swelling of ectodermal cells, is not yet settled. In internal regions the size of cells was normal, but mitotic chromosomes showed vacuolization or delayed separation. The distribution curves of volume of single nucleus were, unlike sodium fluoride, always V-shaped (Table 3).

Sodium azide. About the same concentration (1/10,000 M) was

critical for the gastrulation in lower oxygen concentrations. The injury seems to be cytoplasmic especially for the animal part of the ectoderm. The frequency of the double nucleus and the mitosis was relatively small (Table 4). But mitotic figures were, if present, quite intact. The volume distribution curve of single nucleus was always V-shaped.

DNP. If the concentration was larger than 1/3 mg. %, the cytoplasm of ectodermal cells showed general cytolytic destruction, but the cell-size of the outer layer was similar to that of the internal portion. The nucleus showed spherical condensations. In the critical concentration the double nucleus was abundant and typical, but the distribution curves of volume of single nucleus were V-shaped (Table 3).

Table 5. List of chemicals employed in this study with their effects upon the volume distribution curves of single nucleus

Chemicals	Critical concentration	Shape of the volume distribution curves of single nuclei	
		In oxygen	In nitrogen
Control		V	W
Mono-iodo-acetic acid	5–10 mg. %	V	V
Adenosine-triphosphate	(20 mg. %)	V	V
2, 4-dinitrophenol (DNP)	1/8 mg. %	V	V
Lactic acid	M/400–M/800	V	V
Lithium chloride	M/5–M/10	V	V
Potassium cyanide	over 10 mg. %	W	W
Potassium thiocyanate	100 mg. %	V	V
Sodium azide	10^{-4} M	V	V
Sodium fluoride	5–10 mg. %	V	U
Trypaflavine	1– 3 mg. %	V	–

Discussion

Being guided by the idea that the distribution of nuclei, more precisely, the gradient of cellular size might be controlled by oxygen conditions in various embryonic parts (Sirakami, 1952), the author cultured the embryo under various oxygen concentrations. As shown above, the double nucleus disappeared almost completely with excessive oxygen at 28° C., and the normal mitosis increased simultaneously, probably at the expense of the former. On the contrary, when the embryos were cultured with deficient oxygen or with KCN

at the same temperature, results were completely reversed. Thus it became possible to control the occurrence of double nucleus by altering the oxygen concentration of culture media. Effects of some chemicals upon the frequency of double nucleus under such conditions were investigated.

The results indicate that most of the metabolic poisons caused the re-appearance of double nucleus under high oxygen concentration. In the course of studies, two types of volume distribution curves of single nucleus were observed. In the preliminary discussion, the author proposed an explanation for the significance of these types: the two-peaked curve might suggest that the fission of double nucleus is very rapid and intensive and the one-peaked curve might be found when the fission is very slow.

Although the chemicals employed with excessive oxygen produced abundant double nuclei, they could not change the unimodal distribution of volumes of single nuclei into bimodal or W-shaped ones. This might suggest that the production of double nuclei is not followed by their splitting into daughter single nuclei. However, since mere abundance in oxygen can inhibit the fission of double nuclei in the normal embryo, we must repeat experiments under deficient oxygen concentration, which is assumed to be favourable for the fission. KCN alone caused transformation of distribution curves from V- to W-shapes even under high oxygen concentration. The result seems to agree with our assumption that the fission of double nucleus is accelerated by deficient oxygen supply or by lowered respiration. However, here a possibility of mistaking a cross section of the partner of double nucleus for that of a small isolated nucleus can not be perfectly excluded, since the frequency of double nucleus is sometimes very high.

The abundant production of double nucleus caused by the chemicals might be thus understood as a sign of slight injuries, the cytoplasmic division of cells with double nuclei being arrested.

This explanation is supported by the fact that most of chemicals also produced abundant double nuclei with V-shaped volume distribution curves of single nucleus under low oxygen concentration. For the normal embryo such oxygen condition is assumed to accelerate the fission of double nucleus, because the curve is thereby changed from V- to W-shape.

The effect of LiCl and ATP is worth noticing in this respect. As shown above, LiCl is the only chemical which causes perfect suppression of double nucleus under low oxygen concentration. It might be emphasized here again that LiCl is effective only when it is applied at a certain developmental stage, *i.e.* at earlier phases of the gastrulation, which coincides with the phase of most abundant production of double nuclei.

As will be reported elsewhere, the volume of the neural tube in the tail bud stage decreases when cultured in water under high oxygen concentration or in M/20 LiCl in air. Both treatments cause the common effects on the volume of neural tube on one hand, and on the double nucleus on the other. Possibility might be suggested that the well-known morphogenetic effect of LiCl has some connection with the inhibition of double nucleus in the early gastrula.

The effect of ATP upon the amphibian embryo is sometimes doubted because of the alleged impermeability of surface coat to ATP. In our studies, however, an effect was recognized, which is similar to that of the excessive oxygen in suppressing the activities of double nucleus.

Summary

1. By changing the oxygen condition of culture media, one can control the frequency of amitosis-like double nuclei, which are most abundantly found in *Bufo vulgaris* during the normal course of gastrulation. When sufficient oxygen is present at high temperature, the double nucleus disappears almost completely. With the decrease in the oxygen concentrations frequency of double nucleus is elevated and the volume distribution curve of single nucleus changes from normal unimodal V-shape into bimodal or W-shape.

2. The following explanation of the shape of volume distribution curves of single nucleus was suggested: When the curve is W-shaped, the double nucleus may be splitting actively into two daughter single nuclei; and when the curve is V-shaped, the splitting may be retarded or inhibited, even though there are abundant double nuclei.

3. Many poisons, such as sodium fluoride, mono-iodo-acetic acid, 2, 4-dinitrophenol, potassium thiocyanate, sodium azide etc., produce abundant double nuclei under high oxygen concentration. Most of them seem to inhibit the division of double nucleus even under low

oxygen concentrations, because they never change the distribution curve of single nucleus into the W-shape.

4. Potassium cyanide produces abundant double nuclei under high oxygen tension and at the same time it changes volume distribution curves into W-shapes. This might be a support for our assumption that the double nucleus is a modification of usual mitosis due to the lowered respiration.

5. Lithium chloride seems to suppress the formation of double nucleus at any oxygen condition, because the double nucleus disappears even under the low oxygen concentration and the distribution curves are always V-shaped.

6. Adenosine-triphosphate appears to maintain the V-shaped volume distribution curves of single nucleus under low oxygen concentration.

7. The following interpretation of results was suggested: The double nucleus is a modification of the typical mitosis, based on some special metabolic activities of embryo under deficient oxygen supply; it belongs to the usual mode of cell multiplication in the course of gastrulation, where the increase in cell number is "doubly assured" by typical mitosis and by double nucleus.

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MORPHOGENETIC EFFECTS OF PENTOSE NUCLEOPROTEIN FROM THE LIVER UPON THE ISOLATED ECTODERM*

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INTRODUCTION

The liver tissue of the adult guinea pig induces structures of the archencephalic region, such as eye, forebrain and nose in the ectoderm of *Triturus*-gastrula (TOIVONEN, 1940; TOIVONEN and SAXÉN, 1955). Some attempts were made to find out the chemical component responsible for the regional effect of this tissue. Thus, TOIVONEN (1950) obtained archencephalic induction with fatty acid fraction as well as nucleoprotein fraction of the liver, and KUUSI (1951) suggested in her extensive experiments a possibility that granules rich in pentose nucleic acid might be the archencephalic factor.

For a further analysis of the morphogenetic mechanism of differentiation, it is desirable to isolate from the tissue a single substance having definite regional effect. In view of the facts that neural induction was obtained by microsomes from the frog liver and mouse kidney (BRACHET, 1943) and that spinocaudal induction was obtained by pentose nucleoprotein fraction from the kidney of guinea pig (YAMADA and TAKATA, 1955, 1956), an attempt was made to isolate a pure sample of pentose nucleoprotein from the liver of guinea pig by a technique introduced by KUTSKY (1953) and COHEN *et al.* (1954). The sample was tested for its regional inductive effect on the presumptive ectoderm of the early gastrula of *Triturus pyrrhogaster*.

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tion to Mr. KENZO TAKATA who helped him with the preparation of some of the samples and their physico-chemical analyses.

MATERIAL AND METHODS

Preparation of the Samples

The liver tissue was homogenized with two volumes of 0.14 M NaCl solution by Waring blender or by glass homogenizer. All the procedure was carried out at 0°–5° C. The liver homogenate was stirred continuously for 30 minutes and spun at 18,000 r.p.m. for 20 minutes. The supernatant was recentrifuged under the same condition. An aliquot of this recentrifuged supernatant was precipitated by adding cold 95% ethanol up to the final concentration of 70%. The precipitate was collected by centrifugation, preserved in 95% ethanol at 5° C., and designated as sample *E*.

To the remaining extract M/5 stock solution of streptomycin sulfate adjusted to pH 7.0 was added up to M/50, and the mixture was left standing for 2 hours in an ice bath. The resulting precipitate was centrifuged down. An aliquot of the supernatant was dialyzed in a cellophane bag against M/20 borate buffer of pH 7.6, 0.3 M with respect to NaCl, for about 20 hours with several changes of the external medium. The dialyzed sample was precipitated by cold ethanol as before, preserved in 95% ethanol at 5° C., and designated as sample *SP*.

The precipitate obtained by streptomycin sulfate was gently homogenized with about 50 ml. of M/50 streptomycin sulfate and centrifuged. The precipitate was repeatedly washed from two to four times. The washed precipitate was suspended in 0.3 M NaCl solution of pH 7.6 (M/20 borate buffer) which was about one half the volume of the original tissue used, and gently homogenized. Then it was dialyzed in a cellophane bag against 0.3 M NaCl solution of pH 7.6 for from 15 to 40 hours with several changes of the external medium. The dialyzed suspension was centrifuged and the insoluble material removed. An aliquot of the supernatant was precipitated by cold ethanol and preserved in 95% ethanol at 5° C. This sample was designated as *PT*.

Some Analytical Data of the Samples

Desoxypentose nucleic acid was detected in neither *SP* nor *PT*

by the indole reaction of CERIOTTI (1952). Pentose nucleic acid (PNA) was estimated by measuring the extinction at 260 mμ according to the method of SCHNEIDER (1945) with some modifications. Protein-N in the residue remaining after SCHNEIDER's procedure was determined by the method of LEVY and PALMER (1940). Average PNA content of *SP* was 9.5 PNA-P μg./mg. N, while that of *PT* was 264.0 μg./mg. N. The sample *PT* in its native state showed an absorption spectrum characteristic for nucleoprotein. *SP* contains many protein components as revealed by electrophoresis, while *PT* moves in electrophoretic field as a single component. The details of the physicochemical analyses will be reported in a separate paper.

Operation and Culture

Pieces of ectoderm were isolated from early gastrulae of *Triturus pyrrhogaster*. A piece of the sample prepared as already described, after thorough washing in sterile HOLTFRETER's solution, was inserted between the inner surfaces of two pieces of isolated ectoderm. On an average, the diameter of the isolated ectoderm was about 1 mm. and of the implant about 0.4 mm.

The explants thus obtained were cultured for 10 to 14 days at 18° C. in sterile HOLTFRETER's solution adjusted to pH 7.2–7.3 by sodium bicarbonate. As the control for the reacting system two pieces of isolated ectoderm were fused together and cultured. In 58 control explants only "atypic epidermis" was differentiated in conformity with the earlier observations of YAMADA (1950 a, b).

EXPERIMENTS

Series E: Inductive Effect of Liver Extract

Out of 41 available explants, 38 contained neural structures and 29 of the 38 explants contained structures which were regionally specifiable as archencephalic (Table 1). Of the 29, nine explants contained such structures of archencephalic character as eye-type, forebrain-type and nose-type together. In two explants, eye- and forebrain-type structures were induced together. Although generally the forebrain-type structure was induced close to the implant, when the eye-type structure was induced in addition, the forebrain-type structure developed a little apart from the implant and the eye-type structure always occupied the position closest to the implant.

Table 1. Inductive abilities of liver extract (*E*) and of the components derived from it, pentose nucleoprotein (*PT*) and non-nucleoprotein fraction (*SP*)

Experimental Series	*E*	*PT*	*SP*
No. of available explants	41	78	56
No. of explants with any induction	41 (100%)	78 (100%)	56 (100%)
No. of explants with neural induction	38 (93%)	78 (100%)	33 (59%)
No. of explants containing induction types:			
Eye	15	70	5
Forebrain	25	64	20
Nose	18	62	12
Lens	4	29	1
Pigment vesicle	1	5	2
Non-specifiable brain	7	6	10
Neural epithelium	1	9	0
Placode or neural fragment	15	6	9
Mesenchyme	17	34	40
Melanophore	41	48	52
No. of explants with archencephalic structure	29 (71%)	76 (97%)	23 (41%)

Series PT: Inductive Effect of Pentose Nucleoprotein

Different samples of *PT* always showed strong inductive ability. Out of 78 available explants, 76 contained structures of archencephalic type. Eye-, forebrain- and nose-type structures were induced together in 52 explants, in 27 of which were induced lens-type structures in addition. In all 52 explants, the spatial arrangement of the induced structures was remarkably uniform (Figs. 1 and 2). As stated in the preceding series, the eye-type structure was always induced having the largest area of contact with the implant and the forebrain-type structure developed next to it with or without contact with the implant. In many such instances, the eye-type structure and the forebrain-type structure formed a continuous neural system (Fig. 1). The nose-type structures were not necessarily induced at the side of forebrain-type structures. There were explants in which the nose-type structures were induced at the side of eye-type structures even when the forebrain-type structure was absent. The lens-, or lentoid-type structures were generally induced secondarily by the eye-type structures. However there were explants in which the lens-type structures were induced without direct association with the eye-type

structure (Fig. 2).

Of the remaining two explants containing no archencephalic structure, one had a massive neural epithelium and the other a brain vesicle in addition; but in both cases no structure of regionally specifiable character could be observed (Table 1).



Fig. 1. A large neural system accompanied by nose- and lens-type structures on both sides induced by an implant of pentose nucleoprotein of the liver (Series *PT*). A tapetum-like accumulation of pigment indicating the eye-type differentiation. The neural area away from the implant showing forebrain-type structure (×95).

Series SP: Inductive Effect of Non-nucleoprotein Fraction

Table 1 shows that out of 56 explants, neural induction was observed in 33 of which 23 contained some of archencephalic structures. In the remaining 23 explants, only mesenchyme and/or melanophores could be observed. Eye-, forebrain- and nose-type structures were induced together in only four explants, and forebrain-type and nose-type structures were induced together in seven explants.



Fig. 2. A large eye-type structure induced by an implant of pentose nucleoprotein (Series *PT*) accompanied by lens-type structures and some mesenchyme (×95).

When the inductive ability of the three samples were compared, the differences were evidenced by the induction frequency of each of archencephalic structures. The induction frequency of eye-, forebrain-, or nose-type structure was always higher in Series *PT* than in Series *E*, and was higher in Series *E* than in Series *SP* (for the statistic significance of difference see the explanation in Fig. 3). Further, in connection with the weak inductive ability of *SP* it must be noted that mesenchyme was more frequently induced in Series *SP* than in Series *E* and *PT*. The difference of the induction frequency of mesenchyme was significant between Series *SP* and *E* and between Series *SP* and *PT* (Fig. 3). As to the frequency of melanophore induction, there was no significant difference between Series *SP* and *E* but the difference was significant between Series *SP* and *PT*.

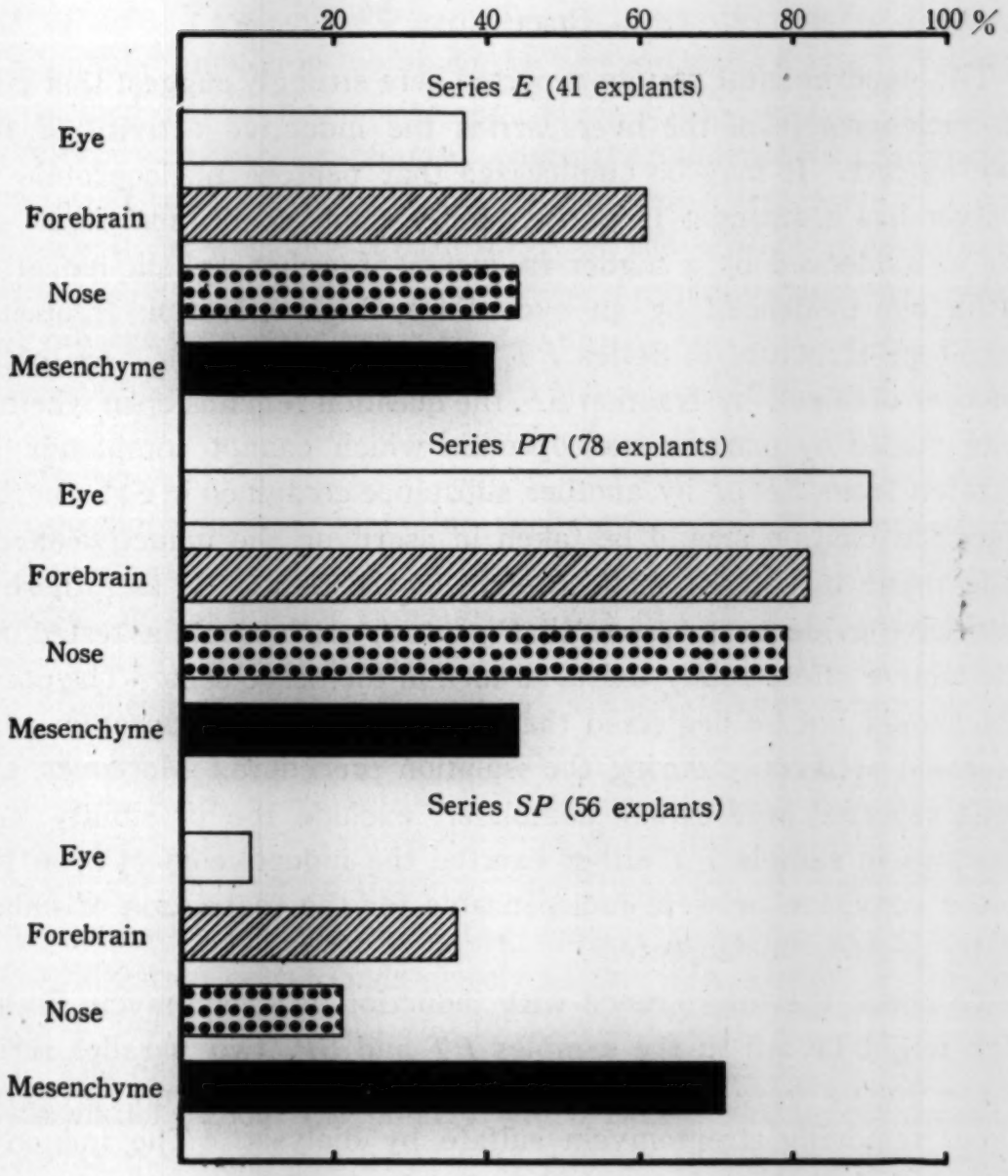


Fig. 3. Diagram for comparison of inductive abilities of different samples. The frequency of induced structures is expressed in percentage of the number of explants containing each structure against the total number of explants in each series. The significance of difference in the induction frequency of each structure was tested by calculating chi-square, and the following values for P were obtained.

Between Series *PT* and *E*, for eye-type and nose-type $P<0.001$, for forebrain-type $P=0.022$, for mesenchyme $P>0.7$.

Between Series *SP* and *E*, for eye-type $P<0.001$, for forebrain-type $P=0.024$, for nose-type $P=0.032$, for mesenchyme $P=0.006$.

Between Series *SP* and *PT* for mesenchyme $P=0.0025$.

Discussion

The experimental results reported here strongly suggest that pentose nucleoprotein of the liver carries the inductive activity of the tissue extract. It may be emphasized that pentose nucleoprotein of the liver has a stronger inductive ability than the original liver extract as evidenced by a higher frequency of archencephalic induction and further evidenced by an exceedingly high induction frequency of eye-type structure in Series *PT* (Table 1 and Fig. 3). As to the induction obtained by fraction *SP*, the question remains open whether it was caused by pentose nucleoprotein which cannot completely be separated from *SP* or by another substance contained in *SP*. In this connection caution should be taken in ascribing the inductive effects of the tissue to pentose nucleoprotein of the tissue. In fact, there is no decisive evidence to prove that pentose nucleoprotein tested for its inductive effect really exists as such in the tissue cells. The possibility should not be neglected that such pentose nucleoprotein may be formed artificially during the isolation procedure. Moreover, the results reported here cannot completely exclude the possibility that impurities in sample *PT* either exerted the inductive effect upon the isolated ectoderm or were indispensable for the realization of induction by pentose nucleoprotein.

To check the interference with induction by streptomycin sulfate which might be left in the samples *PT* and *SP*, two parallel series of experiments were conducted: Sample *PT* and *SP* were prepared without removing streptomycin sulfate by dialysis. The inductive abilities of these samples proved to be almost the same as those of samples *PT* and *SP* from which streptomycin sulfate had been removed by dialysis. Contamination by lipids must also be taken into consideration because inductive ability of lipid fractions from various sources has been proved. However, nucleoprotein fraction composed mainly of desoxypentose nucleoprotein from pancreas, thymus or liver has been proved to be inductive after extraction of lipids by hot alcohol and ether (H. Lehmann, 1936). In a limited series of experiments, *PT* induced large archencephalic structures after repeated treatments with hot alcohol-ether (3 : 1) for 3 minutes.

When neural induction is caused by sub-cytolytic treatment, the structures induced are often in chaotic arrangement (Holtfreter,

1945, 1947; YAMADA, 1950 a). This is in sharp contrast to the archencephalic induction obtained in the present study. The above described distribution pattern (p. 59–60; Figs. 1 and 2) suggests that eye-type structure is induced where the influence of the implant is strongest, while forebrain-type and nose-type structures are formed where it is weaker. Conforming to the earlier observations by YAMADA (1950 b), this is reflected in the finding that the frequency of the eye-type structure is most markedly decreased when the neural induction frequency is decreased (Fig. 3). These facts are also in good agreement with suggestions of TOIVONEN (TOIVONEN, 1951; TOIVONEN and SAXÉN, 1955) and of NIEUWKOOP (1955). The presence of special inducing agents for each archencephalic structure seems to be improbable in view of the fact that sample of pentose nucleoprotein in the present experiments was proved to be almost homogeneous. It is more probable that all the archencephalic structures are induced under the influence of a single factor which might be called the dorsalizing factor (YAMADA, 1950 a, b; YAMADA and TAKATA, 1955) or activating principle (NIEUWKOOP and others, 1952; NIEUWKOOP, 1955). As already pointed out by YAMADA (1950 b), a significant increase in the induction frequency of mesenchyme was observed with a decrease in the general inductive ability (Fig. 3). This situation corresponds closely to the idea earlier arrived at by RAVEN and KLOOS (1945) and by DAMAS (1947).

The experiments reported here were conducted in order to get informations about the process which occurs within the ectodermal cells as their autonomous activity and which leads to archencephalic differentiation. This process, which was called "dorsalization" by YAMADA (1950 a, b), is released by diverse external factors including those of the organizer as well as by sub-cytolytic treatment and indispensable for the occurrence of archencephalic differentiation. The elucidation of this process, indeed, is the cardinal problem for the understanding of the induction phenomena. For this purpose the isolation of homogeneous substance which releases this process and gives rise to typical archencephalic structures at a high frequency must be valuable and indispensable.* It is not the aim of the present

* It might be pointed out that by sub-cytolytic treatment of the ectoderm of *Triturus*-gastrula archencephalic structures can be obtained only in *ca.* 20% of cases, and that the obtained structures are not well organized.

experiments to isolate from the liver the entity itself which is responsible for the induction by the normal organizer. In other words, it is not intended to assert that pentose nucleoprotein of the liver is the very factor which acts normally as the active agent in the organizer. As far as the nature of the active agent is concerned, an immediate and uncritical application of the results obtained by this sort of experiments to the normal organizer action is dangerous, as is shown by the recent experiments of OKAZAKI (1955).

SUMMARY

(1) The 0.14 M NaCl extract of the liver of guinea pig caused archencephalic induction in the presumptive ectoderm of the early gastrula of *Triturus*.

(2) An almost homogeneous sample of pentose nucleoprotein isolated from the same extract induced at a very high frequency archencephalic structures. Inductive ability of this sample was stronger than that of the original extract from which pentose nucleoprotein was derived.

(3) The fraction of the liver extract remaining after removal of pentose nucleoprotein also induced archencephalic structures but not in as high frequency as that induced by the original extract.

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SPINO-CAUDAL INDUCTION BY PENTOSE NUCLEOPROTEIN ISOLATED FROM THE KIDNEY*

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INTRODUCTION

The presumptive ectoderm of the amphibian gastrula has a tendency to form neural structures of archencephalic† type or of regionally nonspecifiable nature under the influence of diverse chemicals including acids and alkalies. However the spino-caudal or deuterencephalic† differentiations have been induced in the same reacting system only by definite regions of the living normal organizer, some adult tissues, or extracts and fractions of the latter (*cf.* KUUSI 1951, 1953), provided that the modifying effect from the host cephalo-caudal niveau is excluded. In our laboratory the microsome fraction of the guinea pig kidney and pH 4.2 precipitable part of 0.14 M NaCl extract from the same tissue have been found to have these regional effects on the isolated ectoderm (YAMADA, TAKATA and OSAWA 1954, YAMADA and TAKATA 1955a). Since both of these fractions contain pentose nucleoprotein (PNP) as their main component, efforts are being made to obtain pure sample of PNP from the tissue. In the present paper the inductive effects of a purified sample of PNP isolated from the kidney of guinea pig are reported and compared with the effects of related fractions of the tissue and of comparable sample

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† DALCQ (1946) introduced the terms, acrencephalon and chordencephalon, to replace archencephalon and deuterencephalon respectively. We adopted the latter terms because of their general use. The expressions, "archencephalic" and "deuterencephalic," are applied in this paper to all regional structures including sense organs and non-neural structures (*cf.* YAMADA and TAKATA 1955a, TOIVONEN and SAXÉN 1955).

of PNP isolated from the liver of guinea pig. Some data of electrophoretic and chemical studies of the used sample of kidney PNP are added.

Technique

Preparation of Samples. The kidney tissue of freshly sacrificed guinea pig was homogenized with two volumes of 0.14 M NaCl. The homogenate was stirred at 0° C. for one hour and then centrifuged at 18,000 r.p.m. for 30 min. To the supernatant was added M/5 pH 7.0 solution of streptomycin sulfate to give the final concentration of M/50. The mixture was left standing for two hours at 0° C. and then centrifuged as above. The precipitate containing pentose nucleoprotein was set aside (see the next paragraph). The supernatant obtained was left standing for *ca.* 14 hours at 0° C. After removing a small amount of the sediment, the supernatant was dialyzed against 0.3 M NaCl adjusted to pH 7.5 with borate buffer for 24 hours, in an ice bath in a cold room. Then cold ethanol was added up to 70%. The sediment obtained was centrifuged down and preserved in 95% ethanol at 5° C. for 3 hours—2 days to be used as implant in the series NF.

The streptomycin precipitate set aside was dispersed in M/50 streptomycin sulfate in 0.14 M NaCl, homogenized gently and centrifuged. The same procedure of suspension and centrifugation was repeated three times. The precipitate finally obtained was dispersed in 0.3 M NaCl adjusted to pH 7.5 with borate buffer, and gently homogenized. The suspension was dialyzed in a cellophane bag against the same solution of sodium chloride under ice cooling in a cold room. After 24 hours of dialysis the solution was centrifuged and the supernatant was used either for chemical and physical studies or for testing morphogenetic effects. For the latter purpose the sample in solution was precipitated by adding cold ethanol up to 70%. The precipitate was collected by centrifugation and preserved in 95% ethanol at 5° C. for 3 hours—3 days for use in series PNP. The procedure was a modification of the technique used by Kutsky (1953) and by Cohen, Levi-Montalcini and Hamburger (1954).

Some Analytical Data. An estimation of pentose nucleic acid was made by a modification of Schneider's method. For the sample

PNP, a mean value of 245.0 PNA-phosphorus μg./mg. nitrogen and for the sample NF, a mean value of 9.0 PNA-phosphorus μg./mg. nitrogen was obtained. According to earlier data, 0.14 M NaCl extract (Fraction A in Tables 1 and 2) contained 18.2 PNA-phosphorus μg./mg. nitrogen. In samples PNP and NF no desoxypentose nucleic acid was detected by the method of CERIOTTI (1952). A typical absorption curve of nucleoprotein was obtained for the sample PNP.

Electrophoretic Data. The sample PNP was studied with a TISELIUS apparatus at pH 8.5 (borate buffer, $\mu=0.135$) and at pH 7.5 (phosphate buffer, $\mu=0.1$). In both cases single boundary was observed. On the other hand sample NF showed many boundaries.

A full account of analytical, electrophoretic, and other data will be given in a separate paper.

Embryological Technique. After complete removal of ethanol the sample was tested on the isolated presumptive ectoderm of early gastrula of *Triturus pyrrhogaster* by sandwich technique (*cf.* YAMADA 1950b and YAMADA and TAKATA 1955a). Each explant contained a piece of sample measuring *ca.* 0.4 mm. in diameter. The explants were cultured in HOLTFRETER solution of pH 7.3 for 9—12 days at 18° C. All explants were studied in sections. Strict aseptic conditions were maintained throughout operation and culture without the use of an-

Table 1. Structures induced in the isolated ectoderm by a sample of pentose nucleoprotein (PNP), non-precipitable fraction (NF), and the original extract (A) from the kidney.

	PNP	NF	A
No. of usable explants	69	60	71
No. of explants with any induction	62	45	65
No. of explants showing the induction specified below			
Archencephalon	0	6	0
Deuterencephalon	34	17	13
Ear vesicle	17	5	16
Somites	21	7	54
Spinal cord	8	2	14
Notochord	3	0	3
Non-specifiable brain	1	4	2
Neural fragment	7	8	10
Mesenchyme	21	19	26

Sometimes one explant contained a number of different structures.

tibiotics. It had been established by a large number of experiments that the isolated presumptive ectoderm of the early gastrula of this species differentiates in HOLTFRETER solution only epidermal cells arranged in an irregular way (YAMADA 1950 b, HAYASHI 1954, YAMADA and TAKATA 1955 a).

EXPERIMENTS

Series PNP: Effect of Pentose Nucleoprotein

Results of microscopic observation are summarized in Table 1. Most of the obtained deuterencephalon-type structures (Fig. 2) were represented by a neural tube winding around the implant, often accompanied by mesenchyme, melanophores and ear vesicle-type structures. The differentiation of the somite-type structures appeared to be retarded and no myofibrils could be detected (Fig. 1). Of the three notochords formed, two were thicker, straight and vacuolized (Fig. 3), while the other was a thin spiral string entangled with a tail neural cord. No structure belonging to the archencephalic region was observed. Thus the sample induced at a high frequency spino-caudal or deuterencephalic structures. As shown in Table 2, the number of explants showing deuterencephalic structures exceeds that of explants showing spino-caudal structures.

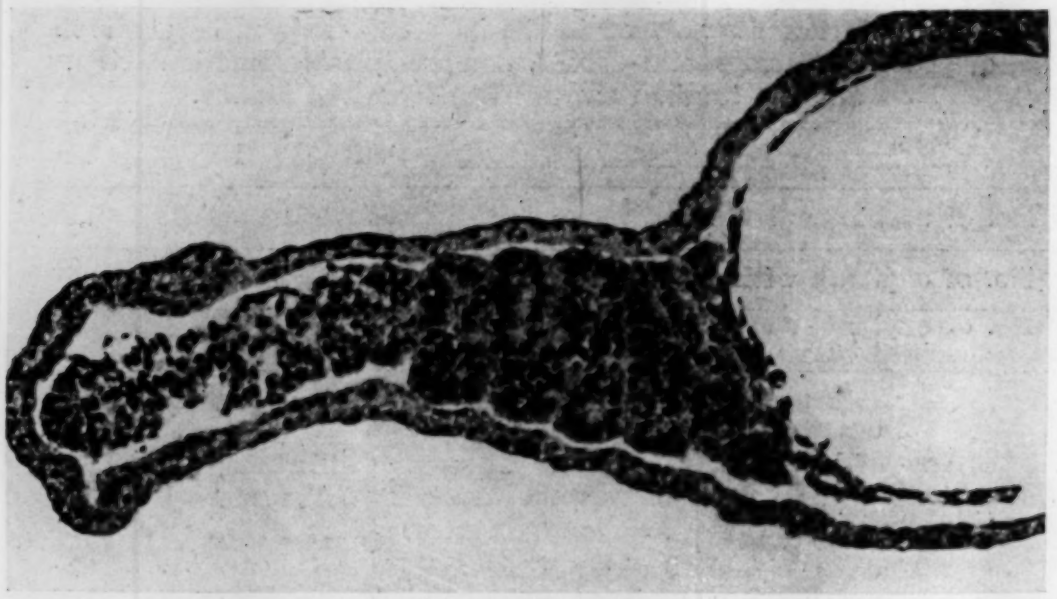


Fig. 1. A tail, containing a series of somite-type structures, and mesenchyme induced by a sample of pentose nucleoprotein from the kidney in the isolated ectoderm. Implant not visible. ×75.

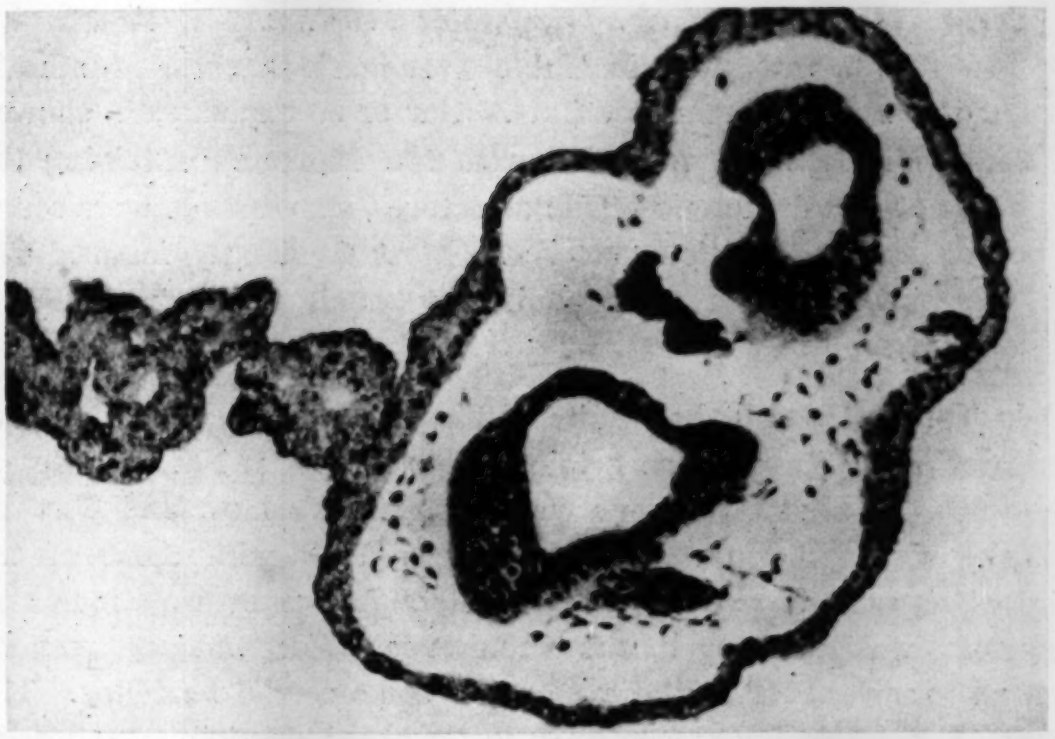


Fig. 2. A deuterencephalon-type structure, accompanied by mesenchyme, and a ganglion-like cell mass induced by a sample of pentose nucleoprotein in the isolated ectoderm. The implant in the middle. ×75

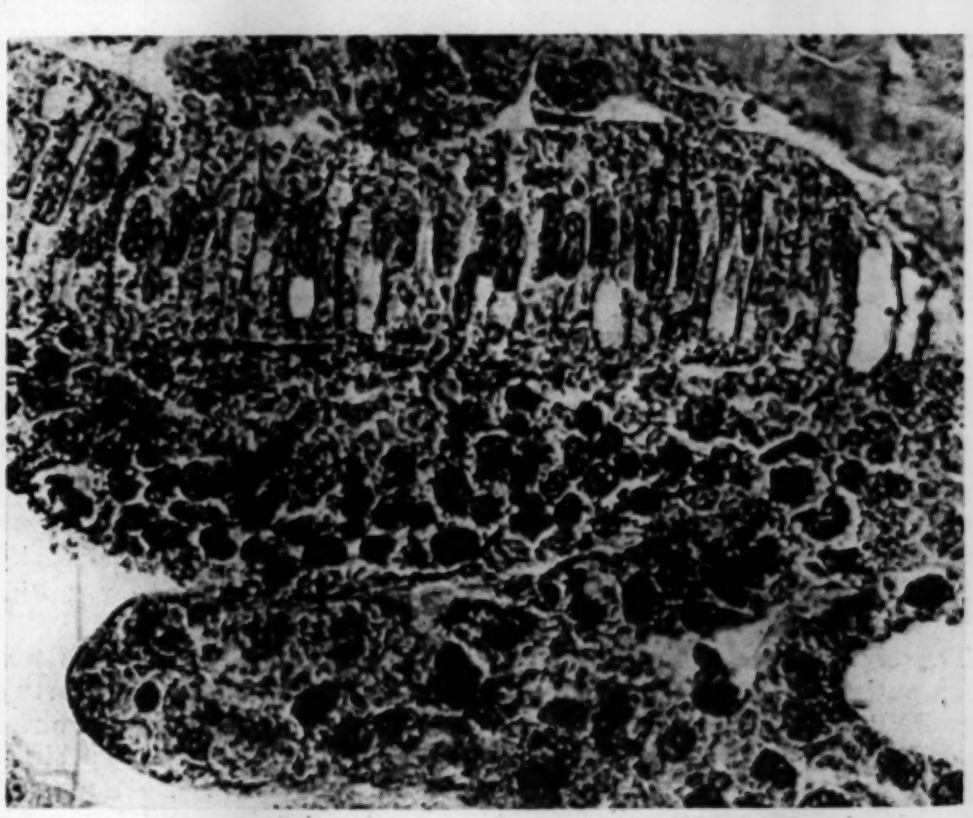


Fig. 3. A notochord-type structure, some neural tissue and a part of ear vesicle-type structure induced by a sample of pentose nucleoprotein (right above) in the isolated ectoderm. ×300

Series NF: Effect of Non-precipitable Fraction

The results presented in Table 1 suggest that the sample is weaker in its morphogenetic effects. Out of 60 explants, 15 showed no definite induction. In addition to the explants containing the spino-caudal and deuterencephalic structures, six explants were found to possess archencephalic structures. Most frequently induced was the mesenchyme often accompanied by small neural vesicle and melanophores.

In Tables 1 and 2 the results of these two series (PNP, NF) are compared with those of the original extract (0.14 M NaCl extract of the tissue, A) taken from our earlier paper (YAMADA and TAKATA 1955 a). Comparing the results of series PNP with those of the original extract, it was found that although the over-all induction frequency was unchanged, the deuterencephalic tendency was increased in series PNP at the expense of spino-caudal tendency. The series NF shows a lower frequency in over-all induction as well as in deuterencephalic and spino-caudal induction, as compared with other series. On the other hand, a weak archencephalic tendency coupled with stronger tendency of non-specifiable neural induction was found in this series. For the statistical singnificance of the frequency difference see the note on Table 2.

Table 2. Regional effects of pentose nucleoprotein (PNP), non-precipitable fraction (NF) and of the original extract (A), expressed in number of explants.

	PNP	NF	A
Archencephalic type	0	6	0
Deuterencephalic type	35	17	23
Spino-caudal type	28	8	59
Non-specifiable	6	16	4
Without induction	7	15	6
Total number of explants	69	60	71

Sometimes one explant contained structures belonging to two different regional types.

The significance of the difference between different series was tested by computing chi-square. The following values were obtained between series PNP and NF: archencephalic, $\chi^2 = 5.157$, $P = 0.023$; deuterencephalic, $\chi^2 = 5.79$, $P = 0.016$; spino-caudal, $\chi^2 = 14.09$, $P < 0.001$; non-specifiable, $\chi^2 = 6.11$, $P = 0.013$; without induction, $\chi^2 = 4.01$, $P = 0.045$. Between series PNP and A: deuterencephalic, $\chi^2 = 4.61$, $P = 0.032$; spino-caudal, $\chi^2 = 25.11$, $P < 0.001$; non-specifiable and without induction, $P > 0.05$.

Discussion

The results presented strongly suggest that the inductive effect of the guinea pig kidney toward spino-caudal and deuterencephalic types as first demonstrated by Toivonen (1940), is carried by PNP of the tissue. In as much as the predominant part of PNP present in the cells is in the microsome fraction, and further, as smaller microsomes contain more PNP than larger ones, the present results are in good agreement with our earlier observation that the smaller microsome fraction caused most frequently spino-caudal induction as compared with other granule fractions and supernatant separated by ultracentrifugation from the kidney tissue (Yamada, Takata and Osawa 1954 a). However, at the present stage of our investigation we cannot insist that spino-caudal and deuterencephalic effects of the tissue are bound exclusively to PNP as separated in the present study, because the non-precipitable fraction used here and the residue of the original extraction with 0.14 M NaCl also have the same regional effects. It is possible but not proven that the inductive effects are bound to the PNP left in these fractions. In any case our results give support to the original idea and experiments of Brachet (1943) as far as the importance of PNP in induction phenomena is concerned. It must be admitted that we have no direct evidence to show that the nucleoprotein here isolated exists as such in the living cells, and it is not an artificial product formed during the isolation procedure. However, for the embryological discussion this point is not of decisive significance, because we are not primarily interested in the physiological significance of this substance in the living kidney cells. Further, we cannot perfectly exclude the possibility that impurities in our sample of PNP were responsible for the obtained induction, because the absolute homogeneity of the sample has not been established.

Earlier experiments with the kidney tissue and its extracts have shown that the factor or factors responsible for the spino-caudal and deuterencephalic induction is quickly inactivated by trypsin and chymotrypsin (Yamada and Takata 1955 b), but is resistant to ribonuclease treatment (Hayashi 1955). On the other hand pentose nucleic acid isolated from the guinea pig kidney failed to show the spino-caudal and deuterencephalic types of induction but occasionally

led to archencephalic or unorganized neural induction (YAMADA, TAKATA and OSAWA 1954 b).

An embryological aspect of the present data appears to be of theoretical importance: The purified sample of kidney PNP induces not a single structure but a number of structures distinctly different from each other within the range of spino-caudal and deuterencephalic types (Table 1). The same set of structures was also induced either by the original tissue (TOIVONEN 1940, YAMADA and TAKATA 1955 a), or by 0.14 M NaCl extract (YAMADA and TAKATA 1955 a) or by microsome fraction (YAMADA, TAKATA and OSAWA 1954 a) under similar experimental conditions. A parallel relationship was found in a comparable series of experiments using the *liver* of guinea pig as the source. Here the purified sample of PNP induced a set of structures belonging to the archencephalic region (HAYASHI 1956). From both experimental series using the kidney and the liver as the source, it can be stated that during the course of isolation and purification of one chemical entity, which was responsible for the induction, no concomitant selection occurred of one special structure out of the array of different structures induced by the original material. On the assumption that the PNP sample used is homogeneous, it can be inferred that the original material induced an array of different structures not because different specific inducing substances corresponding to each structure are contained in it, but because one single substance contained can give rise to all these different structures. If this is the case the initial reaction caused in the ectoderm by a homogeneous sample must be channelled into qualitatively different processes leading to morphologically distinct structures.

Comparing the results of induction experiment with PNP from the kidney and from the liver, we can conclude that the regional character of the induction is dependent upon the difference between the two samples. Kidney PNP brings about differentiation of the spino-caudal and deuterencephalic region, while liver PNP brings about those of the archencephalic region alone. In a sense, this may be a confirmation of the idea that two or more specific substances are responsible for qualitatively different inductions, the idea which was first advanced by HOLTFRETER (1934) and later extended or modified in different directions by CHUANG (1939), TOIVONEN (1940), F. E.

LEHMANN (1950) and others. Data at hand suggest, however, that kidney PNP has the tendency to progressively change its regional specificity, thus inducing more and more anterior structures. This "transformation" is manifested when the sample is left in solution for several days or treated with ethanol, acetate and other chemicals (YAMADA, HAYASHI and TAKATA, unpublished). These facts are in perfect accord with our earlier findings with crude kidney PNP sample (YAMADA and TAKATA 1955 a). The shift of regionality from the spino-caudal to deuterencephalic as observed in the present study between PNP series and the original extract A (Table 2) suggests that the procedure necessary for isolation of PNP from the kidney also has some effect of changing the regionality in the same direction. Further the change in the regional effect of some adult tissues as demonstrated by ENGLÄNDER, VAHS and JOHNEN (1953) and by VAHS (1955) appears to be closely related to this phenomenon. The extensive work of KUUSI (1951, 1953) comprises cases of apparently comparable change in the induction specificity of tissues and their fractions. For instance the brei of the guinea pig kidney with spino-caudal and deuterencephalic effects became an archencephalic inducer after treatments with nitrite, neutral and alkaline formalin (KUUSI 1953). The discussion of these phenomena, especially in connection with ideas suggested by YAMADA (1950 a, b) and NIEUWKOOP (1952) must be reserved for the next publication in which the related data will be given in detail.

SUMMARY

(1) A sample of pentose nucleoprotein was prepared from the kidney of guinea pig by precipitating it with streptomycin sulfate out of 0.14 M NaCl extract of the tissue. The inductive effect of the sample was tested on the isolated ectoderm of the early gastrula of *Triturus pyrrhogaster*. Deuterencephalic and spino-caudal structures were induced in high percentage.

(2) The fraction of 0.14 M NaCl extract which was left unsedimented by streptomycin sulfate was tested for its inductive effect under the similar conditions. A weaker effect comprising archencephalic, deuterencephalic, spino-caudal and regionally non-specifiable induction was observed.

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CHLORETONE AS A PARTHENOGENETIC AGENT IN SEA-URCHIN EGGS

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Chloretone (chlorobutanol or acetone-chloroform) is known as an anesthetic for organisms. This is true for the unfertilized ova of the medaka (*Oryzias latipes*). In the uninseminated eggs of the fish, chloretone (M/200 in M/7.5 RINGER's solution) reversibly inhibits fertilization reaction as well as artificial activation by pricking, chemical and electric stimuli as previously described (YAMAMOTO 1949a, b).

For the purpose of studying the stimulation physiology, an attempt was made to anesthetize sea-urchin eggs with chloretone. A surprising revelation was that chloretone in a sufficient concentration acts as an activator of sea-urchin ova. The reverse effects of chloretone on sea-urchin eggs and fish ova may furnish the interesting problem of the relationship between stimulation and anesthesia in these egg cells.

When the ripe uninseminated eggs of sea-urchins are immersed in chloretone sea water for only a few minutes, typical fertilization membrane forms in the solution and on return to normal sea water the eggs show cleavage. When the eggs which had first been activated with chloretone were subsequently treated with hypertonic sea water and then transferred to normal sea water, they develop into swimming larvae.

MATERIALS AND METHODS

The ripe uninseminated eggs of the sea urchins, *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus*, were used as materials. The spawning season for the former extends from early January to late March and the latter from middle October to late November. These experiments were performed at room temperatures of 11-15° C. for the first and 16° C. for the second. All possible precautions were taken in order to guard against accidental sperm contamination and

experiments were repeated on several occassions.

Concentration of chloretone dissolved in sea water was 0.35 percent (M/50). Since chloretone is not easily soluble in cold sea water, it is advisable to use a water-bath in order to dissolve it in warm sea water. Chloretone must be dissolved and kept in a closed bottle since it is volatile. Because of hydrolysis, the solution is usually acidic. Therefore, pH of the solution is adjusted to 8.2 by adding 1.3 cc. of N/10 NaOH to 100 cc. of chloretone sea water. The chloretone sea water is used after it has been cooled to room temperature.

Results

When the ripe uninseminated eggs are immersed in 0.35 percent (M/50) chloretone sea water (pH 8.2) for 3–5 minutes, the membrane is formed as in normal fertilization. As soon as the eggs are placed in the solution, the roughening process of the surface starts. The fertilization membrane is formed at a point on the egg surface. The process of membrane formation is toward the antipode. The membrane does not always separate smoothly, *i.e.*, it may temporarily adhere to the egg surface at a variable number of points, as in normal fertilization. Finally the membrane becomes completely separated. Typical examples of experiments are shown in Table 1. It may be

TABLE 1. Activation of sea-urchin eggs by chloretone. Unfertilized eggs were treated with 0.35 percent chloretone sea water (pH 8.2) and then transferred to sea water

Time of exposure in min.	*Hemicentrotus pulcherrimus* 11° C.	*Pseudocentrotus depressus* 16° C.
	% of membrane formation	% of membrane formation
0 (Control)	0	0
1	9	2
2	24	15
3	74	22
4	86	90
5	98	96

remarked here that the membrane is formed while the eggs are in the chloretone sea water. When the eggs which were activated with chloretone are transferred into normal sea water, they show irregular cleavage. The rate of cleavage is very slow as compared with that in fertilized eggs. These eggs which are treated with chloretone

alone, can develop only into the morula stage and all further development is arrested. When the uninseminated eggs are treated with 0.175 percent (M/100) chloretone sea water for about one-half hour, only a few eggs are activated. In 0.0875 percent (M/200) chloretone sea water the unfertilized eggs remained intact for a number of hours.

It should be noted that chloretone not only calls forth the membrane formation but also induces the subsequent irregular cleavage. The chloretone-activated eggs reach only the morula stage and none develop into swimming larvae. If, however, chloretone-activated eggs are rinsed in sea wtaer for a while and then treated with the weak hypertonic sea water (sea water 50 cc. + 2.5 M NaCl 8 cc. as originally used by J. LOEB*) for 40–50 minutes and finally transferred to sea water, they undergo relatively normal cleavage. Further development closely resembles that of fertilized eggs although the rate of development is far slower. Development into blastura is reached the first day after treatment, subsequently becoming swimming larvae in the days following. Typical examples of experiments are shown in Table 2. Some larvae develop into normal plutei while others become abnormal. Figs. 1 and 2*A* illustrate parthenogenetically developed *Hemicentrotus* plutei 14 and 22 days old, respectively and Fig. 2*B* shows a parthenogenetically developed 7-day old pluteus of *Pseudocentrotus*. Plutei illustrated in Figs. 2*A* and *B* have well-developed

TABLE 2. Artificial parthenogenesis in sea-urchin eggs induced by double treatment of chloretone and hypertonic sea water. Unfertilized eggs were treated with 0.35 percent chloretone sea water (pH 8.2) for 5 minutes, rinsed in sea water and then put into hypertonic sea water and finally trasnsferred to normal sea water

Time of exposure to hypertonic sea water (min.)	*Hemicentrotus pulcherrimus* 11 °C.		*Pseudocentrotus depressus* 16° C.	
	% of cleavage	% of larvae	% of cleavage	% of larvae
0	19	0	17	0
20	40	0	43	0
30	45	2	49	0
40	51	20	54	19
50	54	36	56	38
60	47	21	49	25

* According to his original papers (LOEB 1905) he used a solution of sea water 100 cc.+ 2.5 m NaCl 15 cc., as hypertonic sea water, but in his two books (1909, 1913) he wrote this formula.

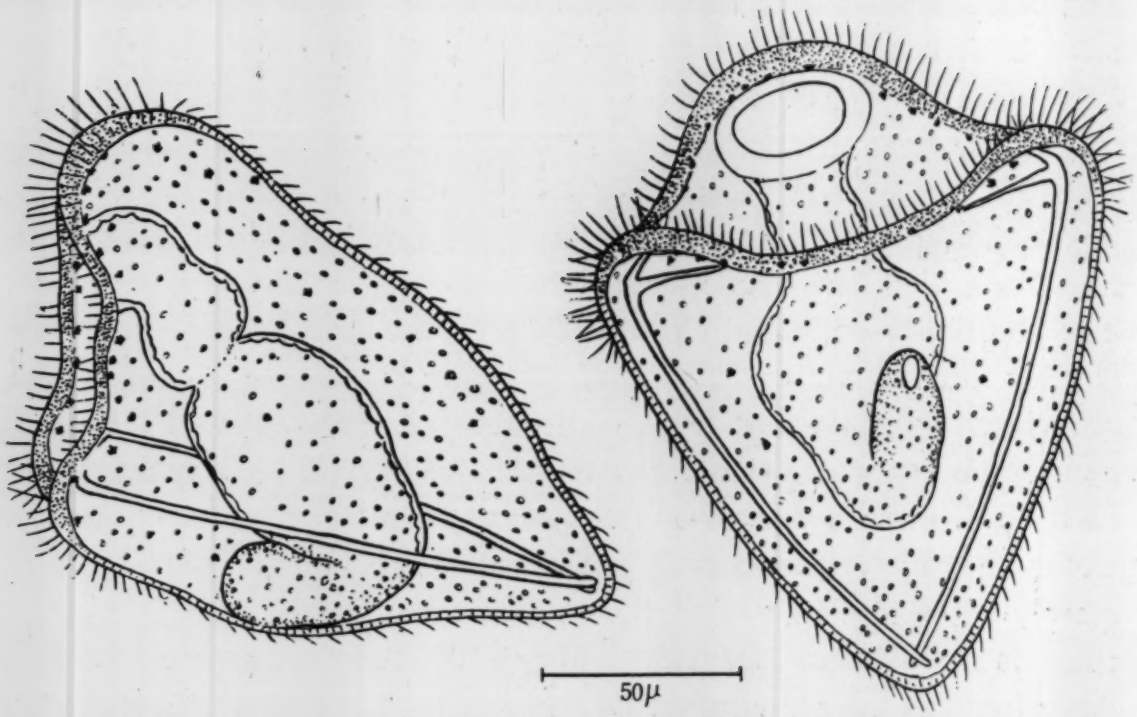
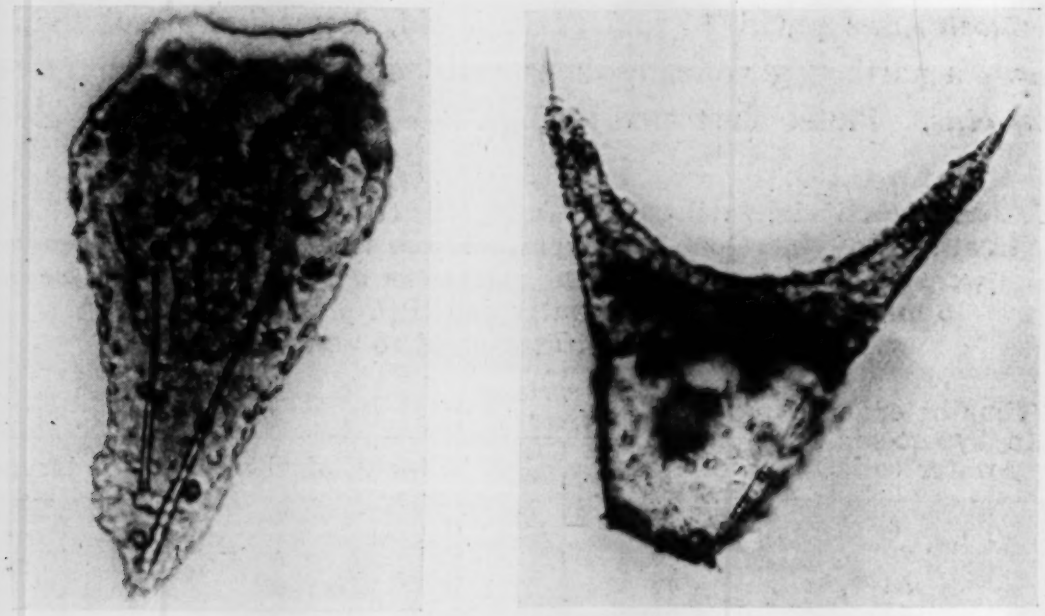


Fig. 1. Lateral and ventral views of a pathenogenetically developed young pluteus (14 days old) of the sea-urchin *Hemicentrotus pulcherrimus.*



A *B*

Fig. 2. *A*: A parthenogenetically developed pluteus (22 days old) of the sea-urchin *Hemicentrotus pulcherrimus.* ×230

B: A parthenogenetically developed pluteus (7 days old) of the sea-urchin *Pseudocentrotus depressus.* × *ca.* 230

arms, mouth, tripartite alimentary canals, anus and spicules. It is clear from these figures that these parthenogenetically developed plutei are close simulations of normals. As already mentioned, their developmental rate is very much slower than that of the fertilized. Mere treatment of uninseminated eggs of these species with the weak hypertonic sea water induces neither the signs of activation nor of cleavage. Therefore it may be concluded that the double treatment of chloretone and hypertonic sea water is responsible for the development of unfertilized eggs into swimming larvae.

Discussion

Chloretone (acetone-chloroform) seems to act upon the lipoid phase of the plasma membrane in such a way as to cause a disturbance which may trigger the chain reactions underlying the membrane formation. The Hertwig Brothers (1887) found that chloroform induces membrane separation in the sea-urchin egg. Herbst (1893) confirmed this and also showed that benzene, toluene, xylene, creosote and clove-oil have the same effect. These agents can call forth only the membrane formation and not the cleavage. Montalenti (1936) found that chloroform induces membrane separation in the eggs of the lamprey, which, when activated with chloroform and subsequently treated with hypertonic salt solutions, develop into morula stage. Loeb (1909, 1913) found that salicyl aldehyde and propyl alcohol bring about membrane formation in the sea-urchin eggs. The writer's experiment (Yamamoto 1951) indicates that chloroform, benzene, ether, toluene and isoamyl alcohol induce complete or incomplete membrane separation in the unfertilized eggs of the medaka (*Oryzias latipes*) depending on duration of the treatment. These experiments indicate that lipoid solvents act as activating agents in eggs of many species. Most of the experiments herein referred to, however, produced membrane formation followed by a final cytolysis. Hence most lipoid solvents are of little value for artificial parthenogenesis.

Chloretone, on the other hand, calls forth not only membrane formation but also induces cleavage, although double treatment in combination with hypertonic sea water is necessary for the development of eggs into swimming larvae. Thus chloretone may be re-

garded as a parthenogenetic agent for sea urchins in the sense of the definition of JUST (1939). He rightly proposed to restrict the term experimental parthenogenesis to cases of activation of eggs which are followed by subsequent development and to exclude all cases of simple membrane formation without subsequent cleavage.

As already mentioned, chloretone acts as an anesthetic for the unfertilized eggs of the fish (*Oryzias latipes*) while exerting stimulation in sea-urchin eggs. The different actions of chloretone on sea-urchin eggs and on *Oryzias* eggs may be explained by the difference in thresholds of stimulation. It is well known that lipoid solvent anesthetics usually call forth a temporary rise in irritability during early stages of treatment (*cf.* WINTERSTEIN 1926, HEILBRUNN 1938). Chloretone may act like lipoid solvent anesthetics on ova. For sea-urchin eggs, a temporary increase in irritability is supposed to be sufficient to evoke the establishment of stimulation while for *Orzias* eggs, it is insufficient. In *Oryzias* eggs, therefore, chloretone does not stimulate, inducing only anesthesia in later stages of treatment.

It is suggested also that chloretone can be used as an agent for experimental analysis of the activation process in sea-urchin eggs because (1) it calls forth membrane formation while eggs are in the solution, (2) it takes some minutes before the cortical changes are completed, and (3) it is harmful only slightly, if at all, to ova as indicated by development of plutei when it is used in combination with hypertonic sea water.

SUMMARY

1. The following is one of the best methods for artificial parthenogenesis in sea-urchin eggs. The uninseminated eggs are first immersed in 0.35 percent (M/50) chloretone sea water (pH adjusted to 8.2) for 3–5 minutes and rinsed in sea water for a while (10–15 minutes) then treated with weak hypertonic sea water (sea water 50 cc. + 2.5 M NaCl 8 cc.) for 40–50 minutes and finaly transferred to normal sea water.

2. Chloretone can be employed as a useful agent for experimental analysis of the activation process in sea-urchin eggs.

3. The apparent reverse effects of chloretone on sea-urchin eggs on which it is an activator and on fish (*Oryzias*) ova on which its

action is to induce anesthesia, are pointed out and discussed on the basis of stimulation physiology.

I wish to express my gratitude to Dr. M. SUGIYAMA of the Sugashima Marine Biological Station for his courtesy during my visits to the station from 1948 to 1950. My thanks are also due to Mr. T. KOBAYASHI, and Mr. M. KATO who kindly made photographs of parthenogenetic plutei.

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CHANGES IN THE EGG MEMBRANE OF THE FISH EGG DURING FERTILIZATION

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In the fish egg, little attention has hitherto been paid to the changes taking place in the egg membrane at the time of fertilization. This may be due to the fact that the egg membrane is already present on the unfertilized egg and is merely elevated upon fertilization. According to the evidence presented by YAMAMOTO (1939-1954), the cortical alveoli embedded in the cortical layer break down upon fertilization and this change is subsequently followed by the elevation of the egg membrane. Experiments on the *Oryzias* egg have indicated that a water soluble colloid is released from the cortical alveoli and that the egg membrane is elevated from the surface by the colloidal osmotic pressure of the alveolar colloid. As to the changes in the egg membrane, it has merely been stated that shortly after fertilization the egg membrane is very soft and gradually becomes hard. Kusa (1949 a, b) has suggested in the salmon egg that the egg membrane hardens as the result of water absorption by the egg and the equilibrium state of the water absorption alters in accordance with the state of hardening. He has also found that the hardening of the egg membrane is chiefly caused by ionic calcium which originates from the body fluid.

The importance of changes in the egg membrane of marine invertebrates at the time of fertilization has been recognized by many workers. MOTOMURA (1941) and RUNNSTRÖM (1947) have shown in the sea urchin egg that the vitelline membrane is transformed into the tough fertilization membrane by the addition of the cortical granule substance. It has also been shown that disulfide bridges and calcium are necessary for the hardening of the membrane (RUNNSTRÖM *et al.* 1943, MONROY and RUNNSTRÖM 1948, HOBSON 1932 and SUGIYAMA 1938).

The present paper deals with some experiments on the changes

occurring in the egg membrane during fertilization of the *Oryzias* egg. It will be shown that the mechanism of membrane hardening in the *Oryzias* egg corresponds closely to that in the sea urchin egg.

Material and Methods

The ripe unfertilized eggs of the orange-red type of the medaka (*Oryzias latipes*) were used as material. The general method of obtaining the eggs has been described by Yamamoto (1944 a). The egg-laying females were isolated from the males the day before the experiments were carried out. The belly of the fish was cut and the ovary was removed and placed in isotonic Ringer's solution. The isotonic Ringer's solution used has the following composition; M/7.5 NaCl 100 parts+M/7.5 KCl 2.0 parts+M/11 $CaCl_2$ 2.1 parts, pH adjusted to 7.3 by adding a small amount of N/10 $NaHCO_3$ (Yamamoto 1939 a, 1944 a). The ripe unfertilized eggs, which can be distinguished from the unripe ones by size and transparency, were isolated with glass needles and kept in Ringer's solution. In overripe eggs, the cortical alveoli break down partially in the ovary and the egg membrane is slightly elevated. These eggs were discarded. A testis was isolated from an adult male, mashed with the blunt end of a glass rod, and diluted with Ringer's solution. Insemination and artificial stimulation were performed in Ringer's solution.

Results

Structure and properties of the egg membrane

It has been shown that the egg membrane of the *Oryzias* egg consists of two membranes: an outer heterogenous or patterned membrane and an inner homogeneous or transparent membrane (Ishida 1944 a). Careful observations reveal that a thin and transparent layer is present outside of these membranes. The outer membrane appears to be tinted faintly yellow. It is less transparent than the inner membrane because of the presence of a fine, beehive-like pattern on its surface. This pattern becomes more apparent after fertilization. The inner membrane appears homogeneous, transparent and colorless. Yamamoto (1944 a) has shown that if the unfertilized egg is immersed in distilled water or in an isotonic solution of urea (M/3.75), the egg membrane swells enormously without the breakdown of the cortical alveoli. This treatment causes swelling mainly

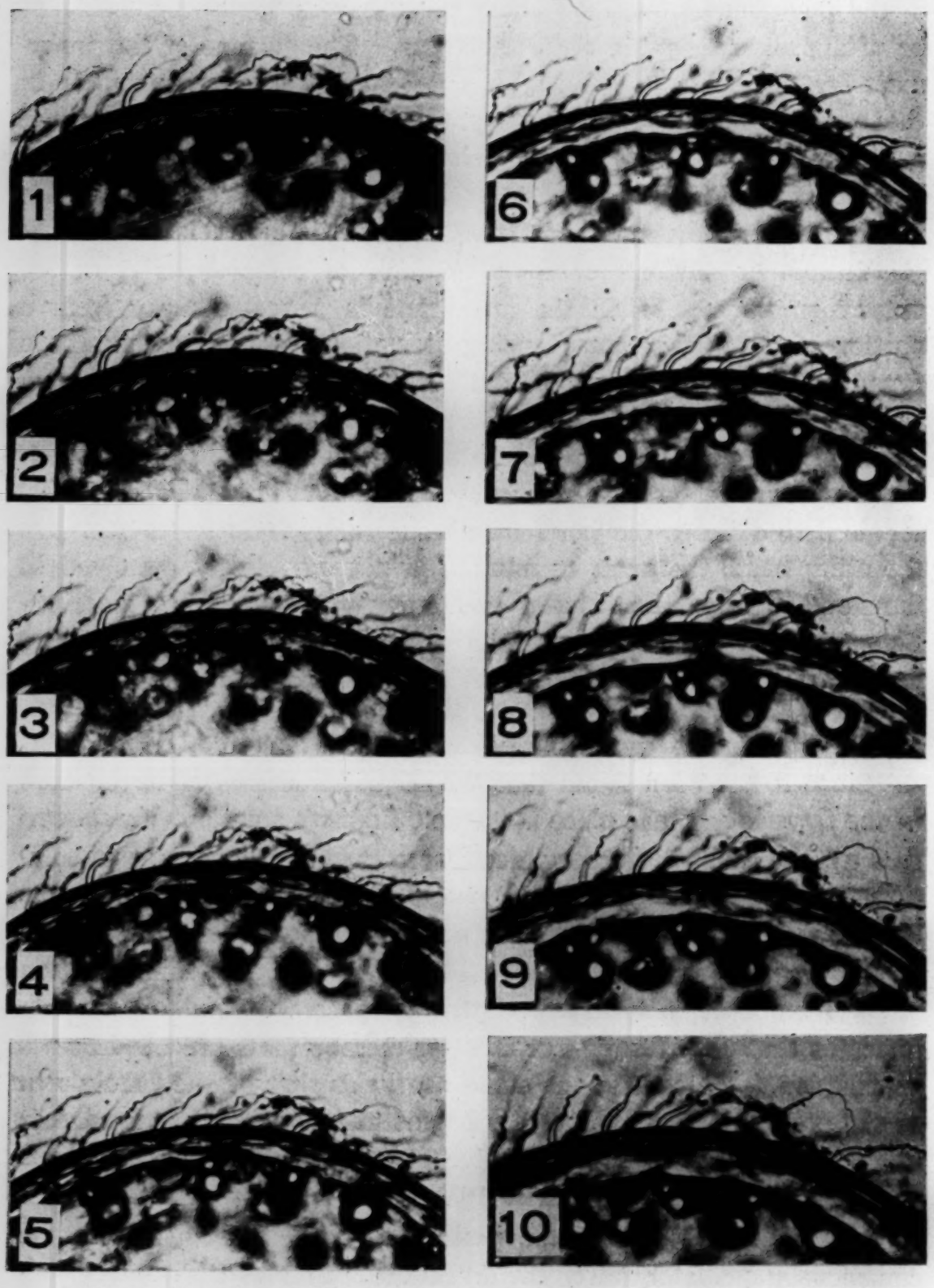
in the inner membrane, and about seven layers are recognizable to be of approximately the same thickness. The surface of the innermost layer appears to be uneven, but this is not distinguishable in the unfertilized egg since the layer is pressed closely against the protoplasmic membrane. As described below, the unevenness becomes apparent immediately after fertilization. Occasionally it is visible in overripe eggs which have been partially activated.

The inner membrane seems to differ in composition from the outer membrane. When the membrane is dissected away from a portion of the egg without causing activation, the inner membrane is found to be very sticky. As AOKI (1941) has reported in the salmon egg, the egg membrane swells enormously in dilute solutions of acid or alkali at room temperature. In alkaline solutions, the inner membrane appears to be dissolved away. The concentrations of NaOH required to dissolve the inner membrane range from 0.1 to 1 M. On the other hand, the outer membrane is not completely dissolved in the same solution. Both inner and outer membranes are more resistant to acid in this respect than to alkali. They are not dissolved in HCl or H_2SO_4 in a concentration as high as 1 N.

Changes in the egg membrane at fertilization

Since it has been found that the normal processes of fertilization in the *Oryzias* egg take place in isotonic RINGER's solution (YAMAMOTO 1939 a), the following observations were made on eggs in this solution. At fertilization the breakdown of the cortical alveoli proceeds in a wave-like manner, beginning near the animal pole and ending at the vegetal pole. This change is followed by the gradual elevation of the membrane to form a perivitelline space. Careful observation indicates that the elevation of the egg membrane is preceded by its contraction. This takes the same course as that of the breakdown of the cortical alveoli, namely, it begins at the animal pole and ends at the vegetal pole. The successive phases of these changes at one point are shown in Figures 1 to 10.

With the exception of the innermost layer, the egg membrane contracts immediately after the breakdown of the cortical alveoli and its thickness rapidly diminishes (Fig. 4). On the other hand, the innermost layer of the membrane swells at first and then gradually contracts. The time course of these changes is graphically shown



Figs. 1-10. Successive changes as seen in optical sections of the periphery of the living egg of *Oryzias*. ×*ca.* 85.

1– 5. 90, 100, 110, 120 and 150 seconds after insemination, respectively.
6–10. 3, 4, 5, 7.5 and 10 minutes after insemination, respectively.

in Figure 11. The points shown in the figure represent averages obtained from 10 observations. The timing varies somewhat with different temperatures, different eggs and different parts of a given egg. It is seen that the contraction occurring in the membrane proper is completed by one to two minutes after insemination. Meanwhile the innermost layer swells and becomes uneven (Fig. 5). It may be noticed that the combined thickness of the two components is rather greater than that of the unfertilized egg membrane. As these changes proceed, the membrane begins to separate from the egg surface (Fig. 6).

In the next stages, the innermost layer contracts gradually and becomes narrower, while the membrane continues steadily expanding (Figs. 7 to 10). This stage lasts about 10 minutes. To complete the contraction, the inner surface of the membrane regains its smoothness and the membrane approaches a uniform thickness. This change is complete by 30 minutes or more after insemination.

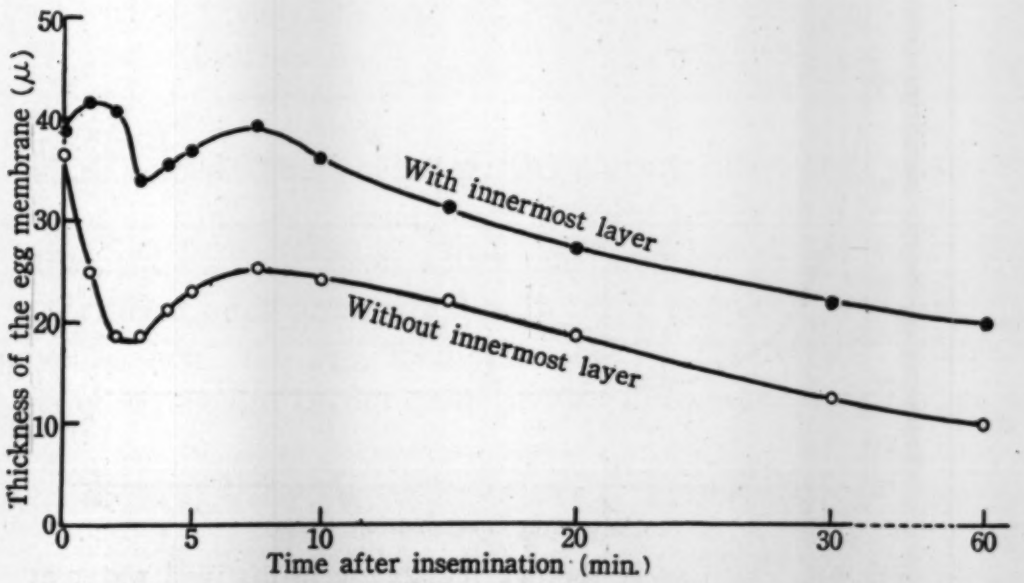


Fig. 11. Changes in the thickness of the egg membrane after fertilization.

It is of interest to note that the hardening of the membrane coincides in time with its complete contraction. When the egg membrane is torn before fertilization, it loses its spherical shape and becomes an irregular mass. If, however, 30 minutes or more have elapsed after insemination, the broken membrane is hard enough to retain its original shape. It appears that the hardening of the membrane is closely associated with its contraction.

It was also found that a once contracted membrane does not swell in distilled water or in an isotonic solution of urea which induces enormous swelling of the membrane before fertilization. If the fertilized eggs are transferred to distilled water at different times after insemination, the effect on the egg membrane depends upon the time after insemination at which the eggs are treated. Thus, as shown in Figure 12, if the eggs are treated with distilled water within 10 minutes after insemination, the membrane swells enormously at earlier stages and the degree of swelling decreases gradually. If, however, the eggs are not transferred to distilled water until 10 minutes after insemination, no swelling takes place. A solution of urea gives rise to the same effect. These results indicate that there is a transitional period during which the egg membrane attains its definitive structure. The egg membrane during this period has a sufficiently low resistance to the reagent so that it is easily affected.

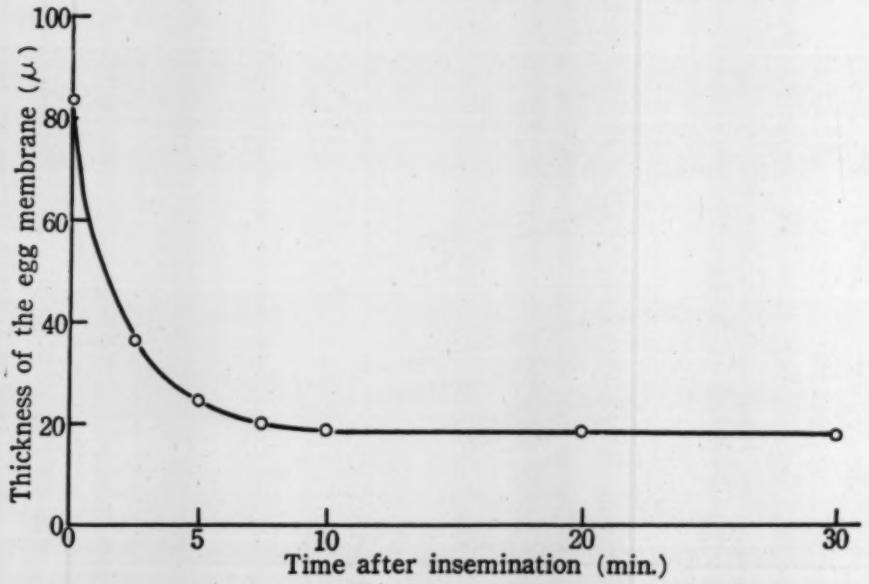


Fig. 12. Swelling of the egg membrane in distilled water at different times after insemination.

In this respect, the egg membrane of the fish egg is comparable to that of the sea urchin egg. It has been found in the sea urchin egg that the fertilization membrane is most sensitive to chemical and mechanical agents during the short period immediately after its elevation (KOPAC 1941).

YAMAMOTO (1944 a) has shown that the breakdown of the cortical alveoli and subsequent elevation of the egg membrane can be induced

by a number of activating agents. Under these conditions, it was also found that similar changes take place in the egg membrane, beginning at the point of the breakdown of the cortical alveoli.

Effect of sulfhydryl compounds on the egg membrane

In the sea urchin egg, RUNNSTRÖM *et al.* (1943) observed that the fertilization membrane is attacked to some extent by sulfhydryl compounds. Similar results were reported by MONROY and RUNNSTRÖM (1948) and these authors considered that the fertilization membrane of the sea urchin egg is keratin-like in nature. In the salmon egg, it was also shown that the egg membrane is a pseudokeratin (YOUNG and INMAN 1938). The possibility was suggested that the membrane of the *Oryzias* egg might be attacked by sulfhydryl compounds.

Table 1. Effect of sulfhydryl compounds on the egg membrane. Solutions made up with isotonic RINGER's solution

Solutions	Degree of swelling of the egg membrane (%)
0.1 M Thioglycolic acid	59.6±3.2
0.1 M Cysteine	67.1±4.4
0.1 M Glutathione	12.6±2.7

In the experiments, cysteine, thioglycolic acid and glutathione were used as sulfhydryl compounds. These reagents were dissolved in isotonic RINGER's solution which was adjusted to pH 7.2. When the unfertilized eggs are treated with 0.1 M solutions of cysteine or thioglycolic acid, the egg membrane swells enormously (Table 1) and many layers are recognizable in the inner membrane (Fig. 13).

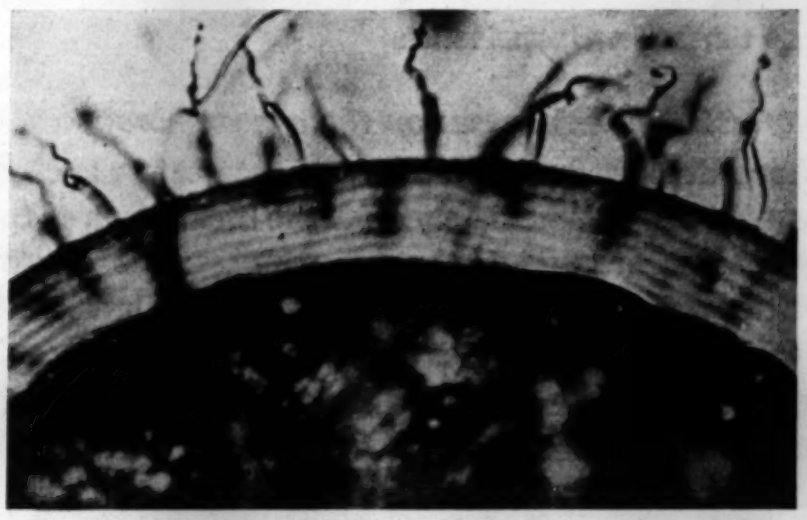


Fig. 13. Egg membrane treated with 0.1 M thioglycolic acid. ×*ca.* 120.

Glutathione is less effective in this respect than cysteine and thioglycolic acid.

In the next experiments, unfertilized eggs were inseminated and exposed to cysteine or thioglycolic acid at different times after insemination. The results of these experiments are shown graphically in Figure 14. It is seen that if time is allowed to elapse after insemination, the effect of the reagents on the egg membrane decreases. In this case too, it is clear that the egg membrane undergoes a transitional period before attaining its definitive structure. This period continues for at least 10 minutes and corresponds to that in the experiments with distilled water.

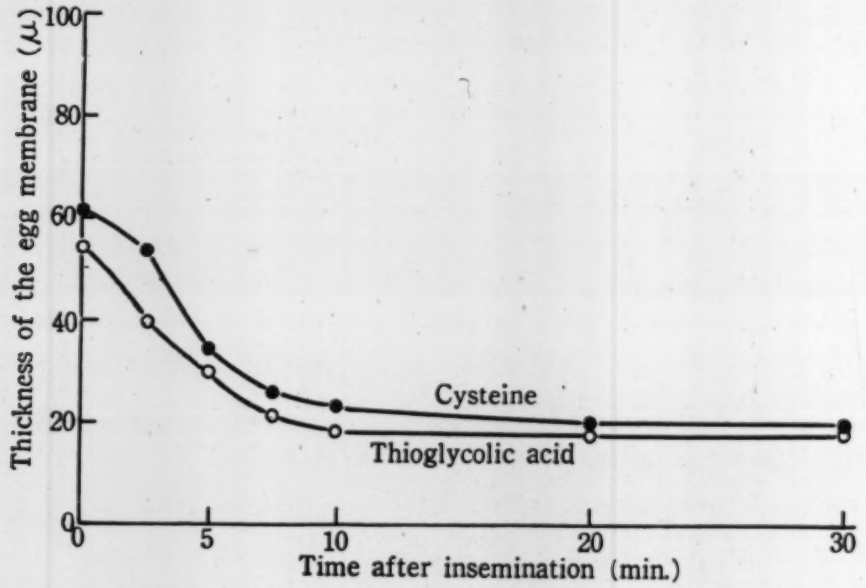


Fig. 14. Effect of sulfhydryl compounds when added at different times after insemination.

Effect of colloidal substances on the egg membrane

The above described observations indicate that the change in the egg membrane begins immediately after the breakdown of the cortical alveoli. On the other hand, if the egg membrane is dissected away from a portion of the egg without causing the breakdown of the cortical alveoli, its hardening does not take place. These facts suggest that the substance of the cortical alveoli plays a role in the change of the egg membrane. According to YAMAMOTO (1939 b), the cortical alveoli contain a water-soluble colloid, to which the egg membrane is impermeable. In order to test the effect of colloidal substances on

the membrane of the unfertilized egg, the following experiments were performed.

Ripe unfertilized eggs were isolated from the ovary and immersed in solutions of colloidal substances such as gum arabic, gum tragacanth, albumin and gelatin. As is shown in Table 2, the egg membranes treated with such colloidal solutions showed contraction to a considerable extent. Under these conditions, complete hardening occurred in the solutions, so that the membrane retained the original shape if the egg was crushed. Even if these eggs were transferred to RINGER's solution or distilled water, the egg membrane remained contracted. This fact suggests that the contraction of the egg membrane is an irreversible process like that occurring in normal fertilization.

Table 2. Effect of colloidal substances on the egg membrane

Solutions	Degree of contraction of the egg membrane (%)
5% Gum arabic	39.3±4.9
1% Gum tragacanth	37.3±5.9
10% Albumin	22.1±4.6
1% Gelatin	38.8±3.9

It should be noted that none of the eggs show visible cortical changes in the colloidal solutions. When eggs treated with the colloidal solution are pricked with a glass needle (15 μ in diameter), the breakdown of the cortical alveoli takes place normally. In these eggs, the elevation of the egg membrane is inhibited, probably as a result of its hardening.

In view of the effectiveness of various colloidal substances in hardening the egg membrane, it is possible that the membrane hardening may be caused, in part at least, by the substance contained in the cortical alveoli. It is of interest, therefore, to ascertain whether the alveolar colloid has an effect on the egg membrane. For the isolation of the cortical alveoli in quantity, the following method was adopted. Ripe unfertilized eggs were washed with cold M/7.5 NaCl (pH 7.2), and centrifuged at 2,000 rpm. for 5 minutes. Under the centrifugal force, the cortical alveoli are accumulated at the centri-

fugal side and the oil droplets at the centripetal side (*cf.* YAMAMOTO 1944 b). This treatment is necessary to prevent the dispersing of the cortical alveoli during the isolation procedure. The eggs were then cut into several fragments in a cold medium. As reported by YAMAMOTO (1951), when the unfertilized eggs were crushed abruptly, activation was inhibited and the cortical alveoli could be obtained in an unbroken condition. Then the disruption of the egg fragments was accomplished by repeatedly sucking and blowing them with a pipette. The cytoplasm and yolk were largely dispersed and the masses of cortical alveoli separated from them (Fig. 15). After the



Fig. 15. Masses of the cortical alveoli. ×*ca.* 40.

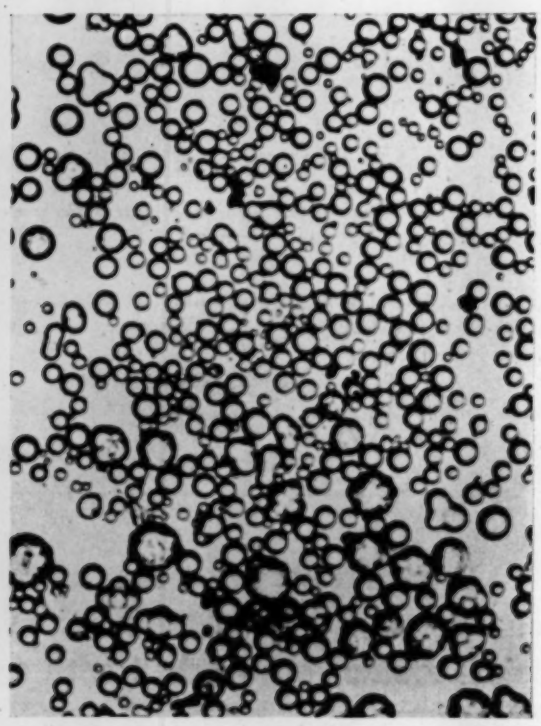


Fig. 16. Isolated cortical alveoli. × *ca.* 50.

egg membranes were removed by centrifugation at 500 rpm. for 1 minute, the masses of cortical alveoli were concentrated by repeated centrifugation at 1,000 rpm. for 3 minutes, and the yolk and cytoplasmic granules which remained in the supernatant fluid were discarded. Finally, the dispersion of the cortical alveoli was aided by repeatedly sucking and blowing with a narrow pipette. The isolated cortical alveoli were washed with M/7.5 NaCl to remove the last adhering cytoplasmic granules. Figure 16 shows the appearance of a typical concentrated suspension of the cortical alveoli obtained by

this procedure. The isolated cortical alveoli are in a condition optically similar to those within living eggs, although some of them form large aggregates. Single alveoli are smooth hyaline spheres, free from granulation when observed under the light microscope. The alveolar wall is readily visible with the phase contrast microscope. Suspensions of cortical alveoli in M/7.5 NaCl may be preserved in the cold for several hours. If, however, they are suspended in distilled water, they break down rapidly and dissolve completely. The aqueous solution of the cortical alveoli shows colloidal properties and gives positive DISCHE, MOLISCH and naphthoresorcin tests. The dissolved substance is precipitated with Janus green and ammonium sulphate at 50 per cent saturation. In typical experiments, 1.12 mg. of the cortical alveolar material was obtained from 860 unfertilized eggs. A detailed report on the chemical nature of the cortical alveoli will be published elsewhere.

For the physiological tests, an aqueous solution of the cortical alveoli was concentrated *in vacuo* and dialyzed against RINGER's solution for 24 hours. When ripe unfertilized eggs were immersed in this solution, the egg membrane showed a typical contraction. The degree of contraction was found to be 31.2 ± 6.6 per cent.

DISCUSSION

The egg membrane of the *Oryzias* egg consists of an outer and an inner membrane. At the time of fertilization, the changes take place mainly in the inner one. Although the origin of these membranes is not known, it may be assumed that the inner membrane corresponds to the true vitelline membrane which transforms into the fertilization membrane. As to the dual structure of the egg membrane, COLWIN and COLWIN (1954) have shown in the enteropneust egg that there are two extraneous transparent coats surrounding the unfertilized egg, and that the inner of these becomes transformed into the fertilization membrane by the addition of material derived from the cortical granules. They have inferred that the inner coat is the vitelline membrane.

The evidence so far available shows that the material of the *Oryzias* egg membrane is a keratin-like protein. ISHIDA (1944 a, b) found that the hatching enzyme, which dissolves this membrane, contains a proteinase. The membrane is also attacked by trypsin and

pancreatin. These results suggest that this membrane consists of a pseudokeratin.

The present experiments show that cysteine and thioglycolic acid induce the swelling of the egg membrane. Inasmuch as the insolubility of keratin is attributable to the disulfide bonds of cystine residues, the swelling of the membrane may be due to their reduction by sulfhydryl compounds. In the sea urchin egg, MONROY and RUNNSTRÖM (1948) have suggested that disulfide bonds contribute to the hardening of the fertilization membrane. It is of considerable interest that the egg membrane is most sensitive to sulfhydryl compounds during the transitional period in which it attains its definitive structure. This period is comparable to that in the sea urchin egg (KOPAC 1941 and KRAUSS 1950).

YAMAMOTO (1939 b) has shown that the egg membrane is elevated from the surface at the time of fertilization by the colloid osmotic pressure of the alveolar substance. Another possible role of the cortical alveoli is that the alveolar colloid takes part in the hardening of the egg membrane. As stated above, colloidal substances, including the alveolar colloid, affect the hardening of the egg membrane. There are indications that this alveolar colloid may be a polysaccharide. It gives positive sugar tests and shows non-dialyzability. Further, the polysaccharide nature of these has been shown histochemically in the salmon egg (KUSA 1954) and in the *Oryzias* egg (AKETA 1954). It is of interest to note that the cortical granules of the sea urchin egg also contain polysaccharide (MONNÉ and SLAUTTERBACK 1950 and NAKANO and OHASHI 1954).

The mechanism by which colloidal substances cause membrane hardening is still a matter of speculation. KUSA (1949 a) has reported a positive effect of gum arabic on membrane hardening in the salmon egg. He also found that membrane hardening takes place on exposure to a hypotonic medium and concluded that the tonicity of the medium plays a role in the change of the egg membrane. However, these cases may be regarded as activation and the membrane may harden as the result of the breakdown of the cortical alveoli. On the other hand, the membrane of *Oryzias* eggs becomes hard in spite of the fact that none of the eggs show visible cortical changes in the colloidal solutions. In this case, colloidal substances provoke the irreversible contraction of the egg membrane, resulting in its hardening.

This phenomenon resembles the so-called supercontraction of keratin molecules, which has been attributed to the cleavage of salt bridges and hydrogen bonds. It seems possible that hydrogen bonds may play a role in the mechanical properties of the egg membrane. However, the possibility remains that the alveolar colloid merges directly with the egg membrane. Unpublished data show that the egg membrane contains about 3.2 per cent sugar. The investigation of this possibility must be the subject of future study. In any case, it is clear that the change in the egg membrane is dependent upon the substance of the cortical alveoli. Probably calcium is also necessary for the hardening of the egg membrane, as shown in the sea urchin egg (HOBSON 1932 and SUGIYAMA 1938) and in the salmon egg (KUSA 1949 b).

It is a pleasure to acknowledge my gratitude to Prof. T. YAMAMOTO for his valuable advice and encouragement. I am also indebted to Dr. J. C. DAN for the critical reading of the manuscript.

SUMMARY

1. The egg membrane of the *Oryzias* egg consists of an outer and an inner membrane. At the time of fertilization, the egg membrane contracts immediately after the breakdown of the cortical alveoli and its thickness rapidly diminishes. This change takes place mainly in the inner membrane. Subsequently the elevation of the egg membrane occurs.

2. Cysteine and thioglycolic acid are shown to induce swelling of the egg membrane, if the eggs are treated within 10 minutes after insemination. This interval corresponds to the period during which the egg membrane assumes its definitive properties.

3. Colloidal substances, such as gum arabic, gum tragacanth, albumin and gelatin, cause hardening of the egg membrane.

4. A method for the isolation of the cortical alveoli in quantity is described. The isolated cortical alveolar material dissolved in distilled water is polysaccharide in nature. After dialysis against RINGER's solution, the alveolar colloid evokes contraction of the egg membrane.

5. The possible mechanism of membrane hardening is discussed.

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STUDIES ON CORTICAL ALVEOLI IN SOME TELEOSTEAN EGGS[1)]

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INTRODUCTION

Fertilization includes this sequence of the events: penetration of spermatozoön into egg interior, contact and, finally, union of the two pronuclei. Thus the egg becomes activated to develop. Besides this type of behavior of the nuclei, fertilization involves far-reaching structural changes in the egg cytoplasm, particularly in the cortical layer of it. Studies of such cortical changes may contribute not only to our understanding of the process of fertilization, but also to the progress in cellular and general physiology, since such spontaneous changes in ooplasm will be intimately associated with a certain aspect of protoplasmic excitation. Although a great body of literature on this subject is now available, most of the papers so far published were concerned on marine invertebrate eggs, on which even exhaustive and comprehensive reviews have recently been given by RUNNSTRÖM (1949, 1951).

In contrast, the problems along these lines were little focussed on vertebrate eggs. The first attempt to demonstrate a cortical change in teleostean eggs is probably that of KAGAN (1935), who found that the "platelets" are eliminated from the cortex of *Fundulus* eggs, upon either fertilization or artificial activation. Just as early as this finding, TCHOU and CHEN (1936), discovered that the "cortical alveoli" disappear from the fertilized eggs of the goldfish, *Carassius auratus*. At the time of these investigations, however, little was yet known as to the physiological nature and significance of the phenomena revealed. Following his discovery of the alveoli in *Oryzias*

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eggs, T. YAMAMOTO (1939–1954) published a series of works on the cortical change in fish eggs.[2] According to him, the most prominent feature characteristic of or essential for the activation of fish eggs is a wave-like breakdown of cortical alveoli, which is believed to be a manifestation of an ooplasmic excitation. He called this excitation-conduction the "fertilization wave". Subsequently to these works, similar structural changes have successively been established in the eggs of many other teleosteans by various investigators.[3]

Now, it appears to be without question that, for the better understanding of such cortical changes in fish eggs, physiological studies of alveoli themselves are very important since these alveoli constitute a basic structure of cortex of various teleostean eggs. In the present paper, the writer wishes to try to give a general summary based on the previous data and some additional results more recently obtained.

Before proceeding further, the writer wishes to acknowledge his indebtedness to Professors T. UCHIDA, T. INUKAI and K. AOKI of Hokkaido University for their unfailing support and interest. His sincere gratitude is devoted to Professor T. YAMAMOTO of Nagoya University for his constant encouragement and valuable criticisms, and particularly to Dr. Y. WATANABE for his helpful advice and generous assistance in preparation of the manuscript. Acknowledgement is also due to the staff of the Salmon Hatchery Stations of Sapporo and Yonezawa, for their collaboration in providing the salmon eggs, and in making the present investigation possible during the years of 1949 to 1954.

MATERIALS AND METHOD

The unfertilized eggs, either fresh or fixed, of the sticklebacks, *Pungitius pungitius*, *P. p. tymensis*, and the salmonid species such as the dog salmon, *Oncorhynchus keta*, the red salmon, *O. nerka* (land-locked form), the rainbow trout, *Salmo irideus* and the brook trout, *Salvelinus fontinalis*, were used as the material for the present investigation. For fixation, several fixatives were employed, such as

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[2] T. YAMAMOTO, 1939a, b, 1944a, b, 1945, 1949a, b, 1950, 1951a, b, 1954a, b.

[3] *Oncorhynchus keta* (KANOH, 1950; K. YAMAMOTO, 1951); *Limanda shrenki* (K. YAMAMOTO, 1951a); *Salmo irideus* (KUSA, 1951; DEVILLERS, THOMOPOULOS and COLAS, 1953); *Triboldon hakuensis* (KANOH and ITO, 1953); *Perca fluviatilis* (THOMOPOULOS, 1953a); *Gasterosteus aculeatus* (THOMOPOULOS, 1953b); *Hypomesus olidus* and *Carassius auratus* (T. YAMAMOTO, 1954b); *Ammdytes tobianus* (THOMOPOULOS, 1954); *Zacco platypes* and *Plecoglossus fluviatilis* (ITO, 1954a).

ALLEN-BOUIN, CHAMPY, REGAUD and alcohol-formol solutions. Some cytochemical tests were also applied. The eggs were inseminated, when needed, after the isotonic method of T. YAMAMOTO (1944a). The RINGER solution used was essentially the same as that first adopted in connection with AOKI's work on salmon eggs (AOKI, 1939), and composed of 100 parts of M/7.5 NaCl, 2.8 parts of M/7.5 KCl and 3.4 parts of M/11 $CaCl_2$, but buffered at about pH 7.0 with a minute quantity of M/10 $NaHCO_3$.

OBSERVATIONS AND RESULTS

Structure of eggs and cortical alveoli

There is no appreciable difference in appearance and characters of the unfertilized eggs between the two stickleback species used, except in number of the eggs produced. The egg is spherical in shape, fairly equal in size in the same batch, with a diameter of 1.0 mm. in average, and coated with relatively thick membrane (*ca.* 10 μ), in which two layers are distinguishable. Close to the undersurface of this membrane lies the egg proper. The histological examination reveals that a thin layer of ooplasm spreads out uniformly over a mass of homogeneous yolk substance, except at the animal pole, *i.e.*, just beneath the micropyle, where the layer is somewhat thickened. A layer immediately under the surface of egg proper is dense with the small "cortical alveoli", each with a diameter of 4



Fig. 1. The phase contrast micrograph of surface of the unfertilized egg of the stickleback (*P. pungitius*). The cortical alveoli appear intensely dark. ×100.

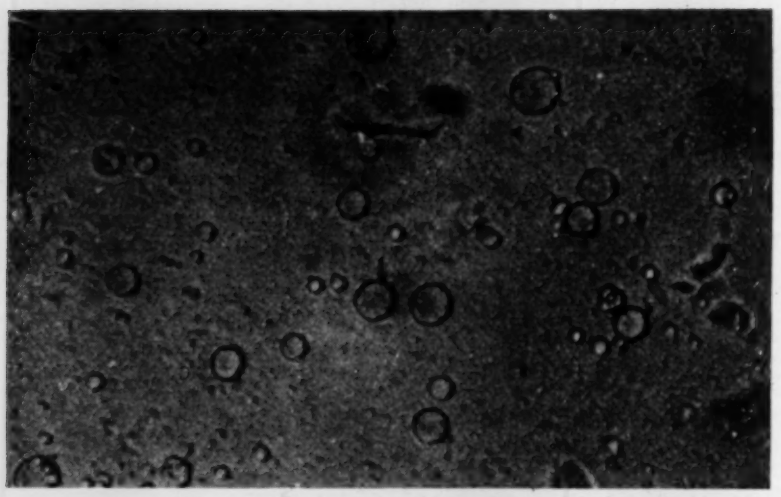


Fig. 2. The cortical alveoli of the stickleback egg, isolated in the Ringer solution. ×200.

to 25 μ. Following insemination or artificial stimulation, the whole system of such alveoli disappears from the egg, and a wide perivitelline space is formed, separating the egg membrane from the egg proper. In the fresh eggs, the cortical alveoli are highly opaque in appearance, but discernible with no difficulty even under low magnification of ordinary microscope. Under the phase contrast illumination, the alveoli appear dark, as shown in Figure 1. With the use of fine needles, they can be isolated in the RINGER solution. The alveoli thus isolated are rather high in density and readily settle on the bottom of the container, with some tendency to be deformed (Fig. 2). Under moderate centrifugation of the entire eggs, the alveoli are displaced toward the centrifugal end. This indicates that the alveoli consist of relatively heavy substance within the ooplasm.

In contrast to the stickleback eggs, the salmonid eggs are much larger in size: the dog salmon egg is about 6 mm. in diameter, and other salmonid eggs, 3 to 5 mm. The egg membrane consisting also of two layers is rather thick, and closely encloses the egg proper, as in the stickleback eggs. Among salmonid eggs, the presence of the cortical alveoli and their breakdown were demonstrated first by KANOH (1950) and K. YAMAMOTO (1951b) in the fixed dog salmon eggs. The alveolar system in the egg surface of other salmonid species can also be observed with similar techniques. In all of these eggs, however, we had still scanty information as to the properties of the fresh alveoli. As a matter of fact, in the intact fresh eggs, the alveoli can

hardly be seen. This difficulty may be attributed rather to the presence of a large quantity of yolk substance than to the opacity of the egg membrane. When yolky fluid is made to diffuse out in the RINGER solution by cutting the egg, the alveolar structure becomes visible on the egg surface (Fig. 3*a*). The alveoli thus seen are somewhat refractive, and appear as bright spots, when placed slightly out of focus (Fig. 3*b*). Under the phase contrast (dark), they look dark

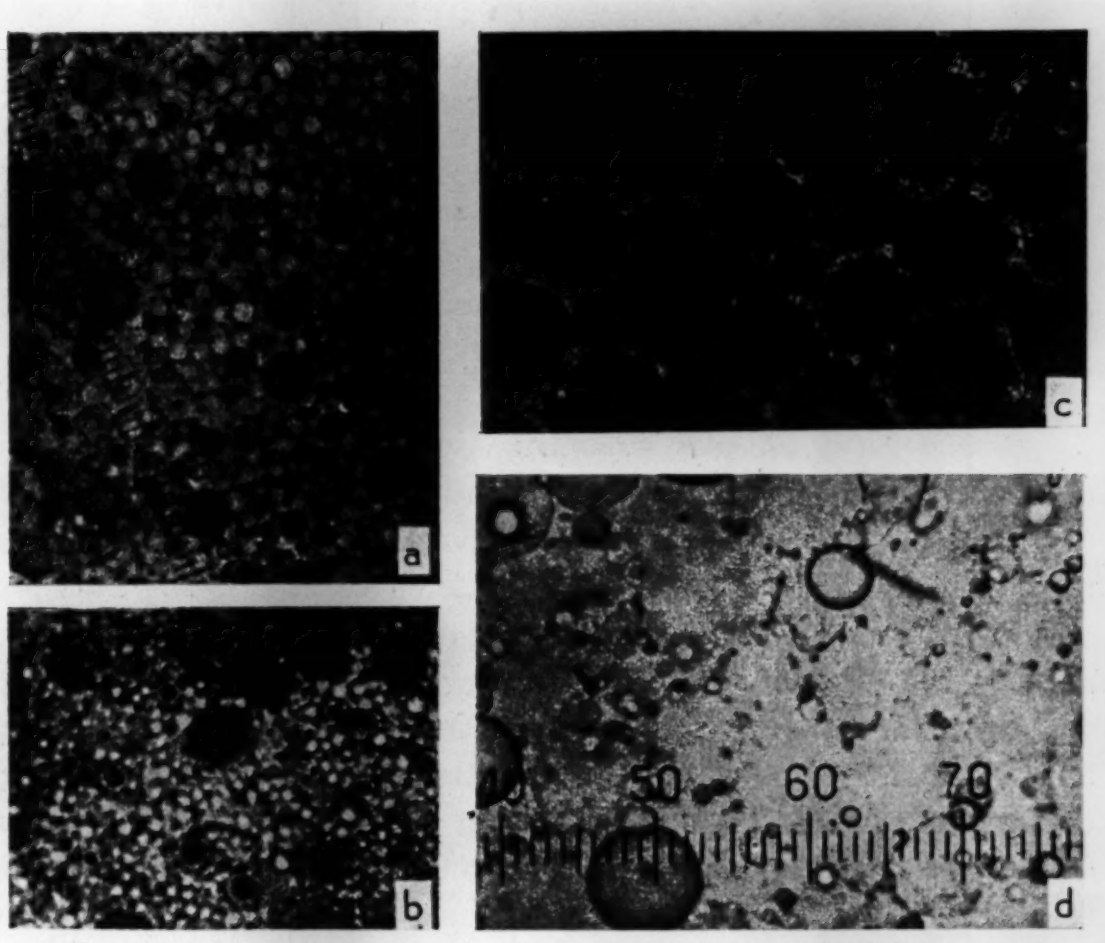


Fig. 3. Alveolar structure in the surface piece, excised from the dog salmon egg in the RINGER. *a*, *b* and *d*, ordinary photomicrographs and *c*, phase contrast. *a*, *b*, × 100; *c*, *d*, × 600. (KUSA, 1954). The explanations are given in the text.

(Fig. 3*c*). Though not frequently, they are seen to coalesce into larger irregular masses. In the interstices between the alveoli, finer inclusions are dispersed (Fig. 3*d*). Recently, the writer has succeeded in isolating the cortical alveoli in the RINGER solution from the trout eggs (Fig. 4). Most of the cortical alveoli isolated in the RINGER are fairly stable for a considerable length of time, but a few burst spon-

taneously. Upon such spontaneous burst or artificial puncture by a needle, enveloping membrane becomes distinctly visible for a while which sooner or later disappears.

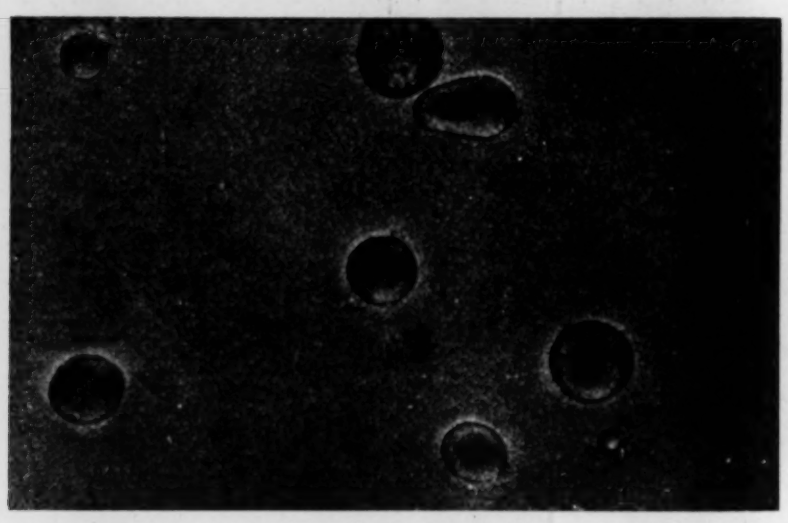


Fig. 4. The cortical alveoli of the rainbow trout egg, isolated in the RINGER solution. ×500.

Breakdown of alveolar structure

General response on the egg surface. When stickleback eggs are fertilized, the breakdown of cortical alveoli occurs from the animal to the vegetal pole in a wave-like fashion (KUSA, 1953b). This process is completed within three minutes or thereabouts at the temperature of 18° C. Soon after this, the egg membrane is progressively separated, and then the blastodisc is raised, where the cleavage occurs later. Similar events are observed after the artificial stimulations. For instance, when the unfertilized egg is pricked with a fine glass needle, the breakdown of cortical alveoli begins at the site of puncture, progressing toward its exact antipode. However, development of the egg artificially stimulated does not advance beyond the blastodisc stage, at least so far as the writer's experience is concerned. In connection with this breakdown wave, it seems of particular interest to note that the rupture of any single alveolus is not always successively followed by the rupture of their neighbouring alveoli. Strictly speaking, the breakdown of alveoli appears to occur rather individually and even at random (Fig. 5). In spite of it, the alveolar structure as a whole breaks down and disappears in a wave-like fashion from one pole to the other on the egg surface. Consequently, the wave-like propagation of the alveolar breakdown should be taken as

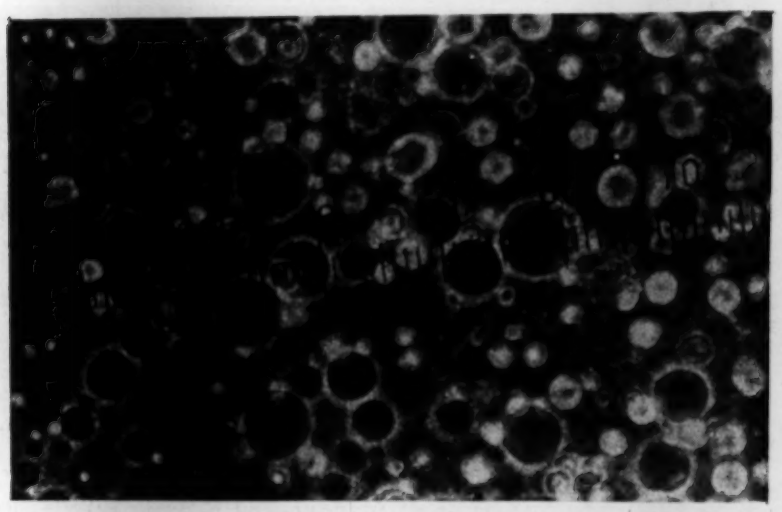


Fig. 5. The phase contrast micrograph of a part of surface of the inseminated stickleback egg. The intact alveoli appear dark in shade and those under breakdown, whitish. ×300.

a statistical or collective manifestation of individual processes. The wave of breakdown will be a secondary event caused by a certain invisible change taking place all over the ooplasm upon egg activation. This view may well stand in harmony with T. YAMAMOTO's theory, in which the "fertilization wave" is supposed to be a sort of protoplasmic impulse propagating on the egg surface prior to the alveolar breakdown (T. YAMAMOTO, 1944b).

When sufficiently exposed to Ca-free RINGER, or to Na-oxalate solution which is a strong Ca-precipitant, the stickleback eggs (and the salmonid eggs as well) become insensitive to insemination, but completely recover their fertilizability, when well rinsed with normal RINGER. Under a Ca deficiency the pricking also becomes ineffective for the activation of the stickleback eggs. But when the eggs pricked in Ca deficient media are returned to normal RINGER solution, the cortical change takes place quite normally with no additional pricking. Similar reversible block is also brought forth with narcotics, such as chloretone. After exposure to 2 per cent chloretone in the RINGER, no cortical change occurs in the eggs either on insemination or on artificial stimulation. But, when rinsed with the normal RINGER, these eggs completely restore their susceptibility to the stimulating agents. Such reversible inhibition of the cortical change in stickleback eggs is very similar to that in *Oryzias* egg (T. YAMAMOTO, 1944a, 1954a).

Alveolar breakdown occurs spontaneously in salmonid eggs on contact with distilled water as well as with fresh water, hypotonic RINGER and other salt-free media (KUSA, 1950). Even after sufficient exposure to Ca-free RINGER or to narcotics, the eggs of dog salmon are readily activated in distilled water, with the cortical alveoli broken down, and invariably take up water, though fertilization is still reversibly blocked. Thus, the absence of Ca ion from the media appears to have little to do with the cortical breakdown[4].

Structural change in individual alveoli at the breakdown. Although

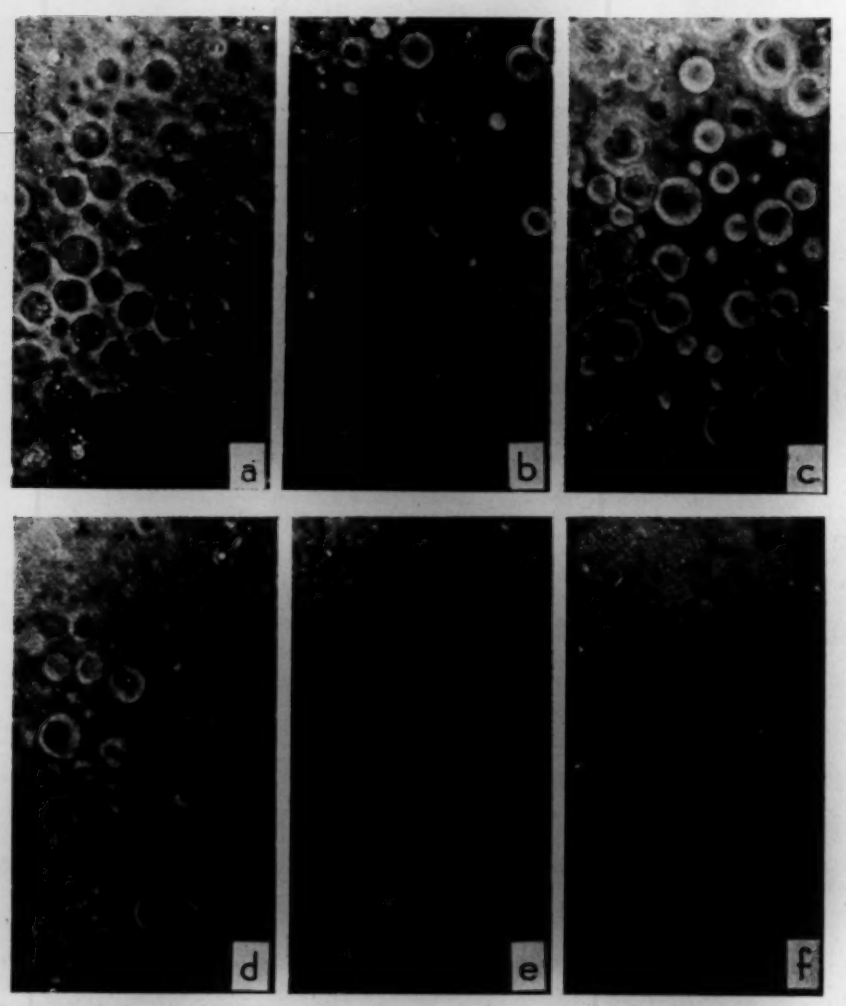


Fig. 6. The phase (dark) contrast photomicrographs of serial changes of alveolar breakdown in a certain region of the egg surface. *a*, immediately, and *b-f*, at 2'50", 3'10", 3'25", 3'45", 4'10" after insemination, respectively. ×300. (KUSA, 1953 b)

4) For the fuller account of the rôle of Ca ion in fertilization and artificial activation, the recent paper by T. YAMAMOTO (1954 a) may be much consulted.

considerable work has been done on the breakdown of the cortical alveoli in teleostean ova, the structural change accompanying the breakdown has not yet been fully clarified. Fortunately, for detailed observation of the breakdown process, the stickleback alveoli are better suited (KUSA, 1953b). At the beginning of the breakdown, the alveoli suddenly become hazy in outline, but soon after much brighter and larger in size, each showing double circles on the surface. A few seconds later, they shrink and finally disappear from the sight. Phase contrast microscopy makes it clear that the breakdown of alveoli is accompanied by a change from dark to light in shade. The photomicrographs in Figures 6 and 7 illustrate these successive stages of breakdown in individual alveoli of the fertilized stickleback eggs.



Fig. 7. The phase contrast micrographs of the stickleback alveoli. *a*, intact alveoli, *b*, the same at maximal swelling and optical change before breakdown. ×300. (KUSA, 1953 b)

In the salmonid eggs, the breakdown process is essentially the same as that in the stickleback eggs. After adequate treatment with fresh water, spontaneous activation can be well observed in the dog salmon eggs even in the RINGER solution (KUSA, 1950). The observation on pieces of the surface excised from such eggs reveals that a majority of the alveoli becomes much brighter, each with double circles on the surface, in some of which active shrinkage is clearly seen.

A more accurate picture of such a change in individual alveoli can be obtained by a optical section of the stickleback eggs. A diagramatic illustration is given in Fig 8:—Preceding the actual breakdown (*A*), the alveolus abruptly becomes vague in appearance (*B*), but soon forms an aperture-like structure facing onto the egg surface (*C*). At this stage, the alveolus is actively retracted, probably due to regional contractions of the egg surface. Thereafter, the content is forced out (*D*, *E*) and at last lost to the egg (*F*). Judging from the sequence of these changes, it is apparent that increase in volume

and optical change associated are involved in the stage *C*, and the inner ring of the image of alveolus may represent the cross section of the aperture. Subsequently to the stage *E*, it shrinks into a polyhedral form. After these changes, the egg membrane is separated from the egg. In consequence, it may be pointed out that, upon activation, the content of the cortical alveoli is actually extruded from these egg.

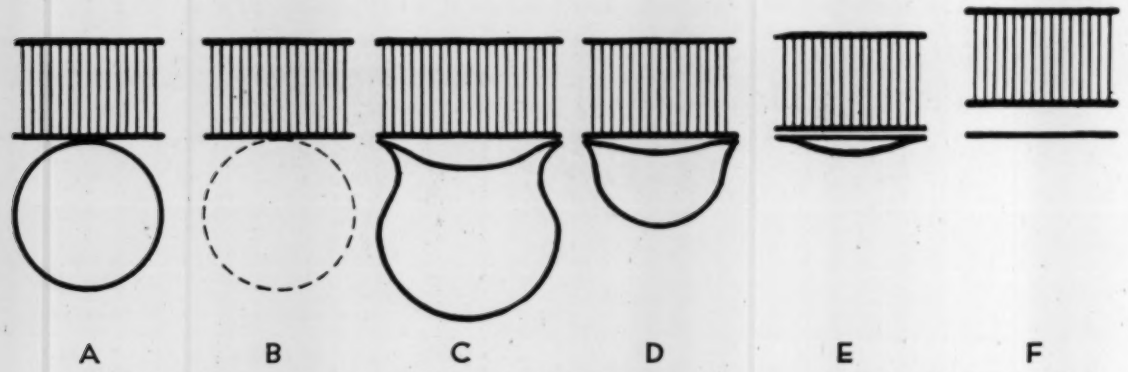


Fig. 8. Diagrams illustrating the breakdown process of individual alveoli (radial view). (KUSA, 1953 b). The explanations are given in the text.

Chemical nature of alveolar contents

It was generally believed that the cortical alveoli contain colloidal substance, but no conclusive evidence had yet been coming forth. Herewith, to determine the chemical nature of alveolar content, several staining tests were tried on the sections of fixed materials (KUSA, 1953 b, 1954). The alveolar contents in the eggs of dog salmon and red salmon stain with various basic dyes, such as thionin, neutral red, toluidine blue, safranin 0, dahlia violet, Janus green B and DELAFIELD's hematoxylin (Fig. 9). The first four yield much sharper stain than the others. But the alveoli have little affinity for acid dyes, such as eosin, acid fuchsin and orange G, except aniline blue in MALLORY's triple stain. With some basic dyes, the alveoli exhibit distinct metachromasia: reddish violet with both thionin and toluidine blue, and brown with neutral red. Usually heparin and other sulfated polysaccharides are known to produce intense metachromasia (LISON, 1936; GOMORI, 1952). Under some conditions glycogen becomes metachromatic (GOMORI, 1952), but the alveoli do not seem to owe their metachromatic properties to the presence of glycogen, for the alveoli are found entirely negative to the iodine reaction. It is highly probable that, in the salmonid alveoli, sulfated polysaccharide is con-

tained. This is reasonably inferred from their affinity for the aniline blue in MALLORY's triple stain for mucus, and also substantiated by the stainability and metachromasia with pyronin B solution aged 50 days at the room temperature. According to MONNÉ and HÅRDE (1951), the affinity for aged pyronin is another feature of sulfated mucopolysaccharide.

Such polysaccharide nature of the alveolar content is also evidenced with the HOTCHKISS test (Figs. 10 *a* and *b*). This cytochemical reaction involves oxidation of vicinal hydroxyl groups to the aldehydes by means of periodic acid, and subsequent coloration of the aldehydes with SCHIFF's reagent (*cf.* GLICK, 1949). With the oxidation by means of periodic acid omitted, no coloration occurs in the cortical alveoli. To BAUER's CrO_3-SCHIFF's test, in which chromic acid is used as oxydant, the alveoli are equally positive. However, hyaluronic acid is probably absent within the alveoli, since their basophilia remains quite unchanged, even after 2 to 5 hour treatment with hyaluronidase at the temperature of 37° C. This basophilia is not due to the presence of ribonucleic acid. It is unaffected by 30 minutes' treatment with 4% trichloracetic acid at 90° C., in which ribonucleic acid should be removed from the preparation (SCHNEIDER, 1945). The FEULGEN test for desoxyribonucleic acid is negative too. The protein nature of the salmonid alveoli is yet uncertain. With ninhydrin-SCHIFF's reagent (YASUMA and ICHIKAWA, 1951), the alveoli give a slightly positive reaction as compared with yolk mass (Figs. 10 *c* and *d*). The examination with use of mercuric bromphenol blue reaction (MAZIA, BREWER

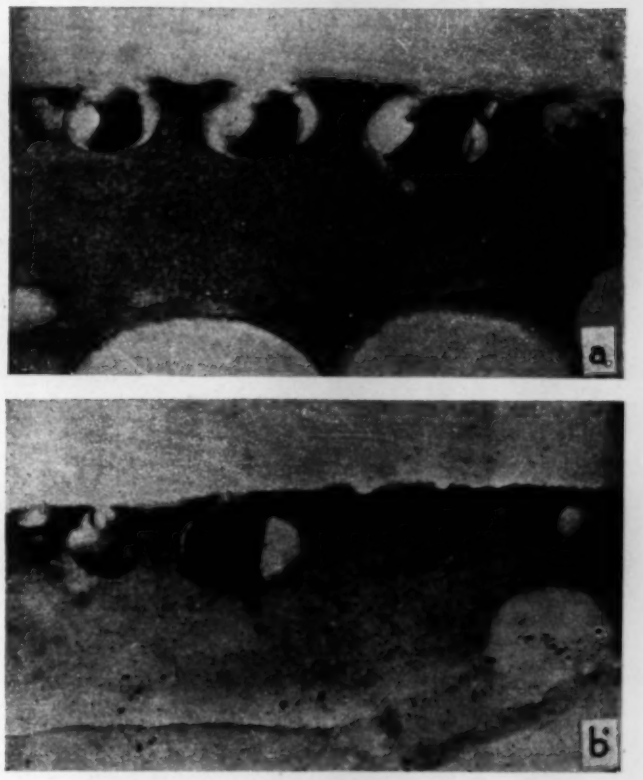


Fig. 9. The histological preparations of cortical cytoplasm of the unfertilized dog salmon eggs. *a*, stained with neutral red; *b*, with pyronin B. ×450 (KUSA, 1954)

and ALFERT, 1953) proves that the alveoli are scarecely positive.

A similar polysaccharide substance is found within the cortical alveoli of the stickleback eggs (KUSA, 1953 b). The alveoli, fixed with GILSON or BOUIN fixative, are positive for the HOTCHKISS test (Fig. 11), and stain well with the aniline blue in MALLORY's triple stain, but they have no affinity for the BEST carmine (for glycogen) whatever. The cortical alveoli isolated in the RINGER solution also vitally stain with various basic dyes; particularly with toluidine blue, they exhibit metachromasia.

In the alveoli of these two groups of eggs, however, no lipoid substance can be detected, either with CIACCIO test or by osmium impregnation.

Mitochondria-like particles attached to cortical alveoli

The histological examination has proved in the stickleback eggs, fixed with CHAMPY's fluid, that minute particles, stainable

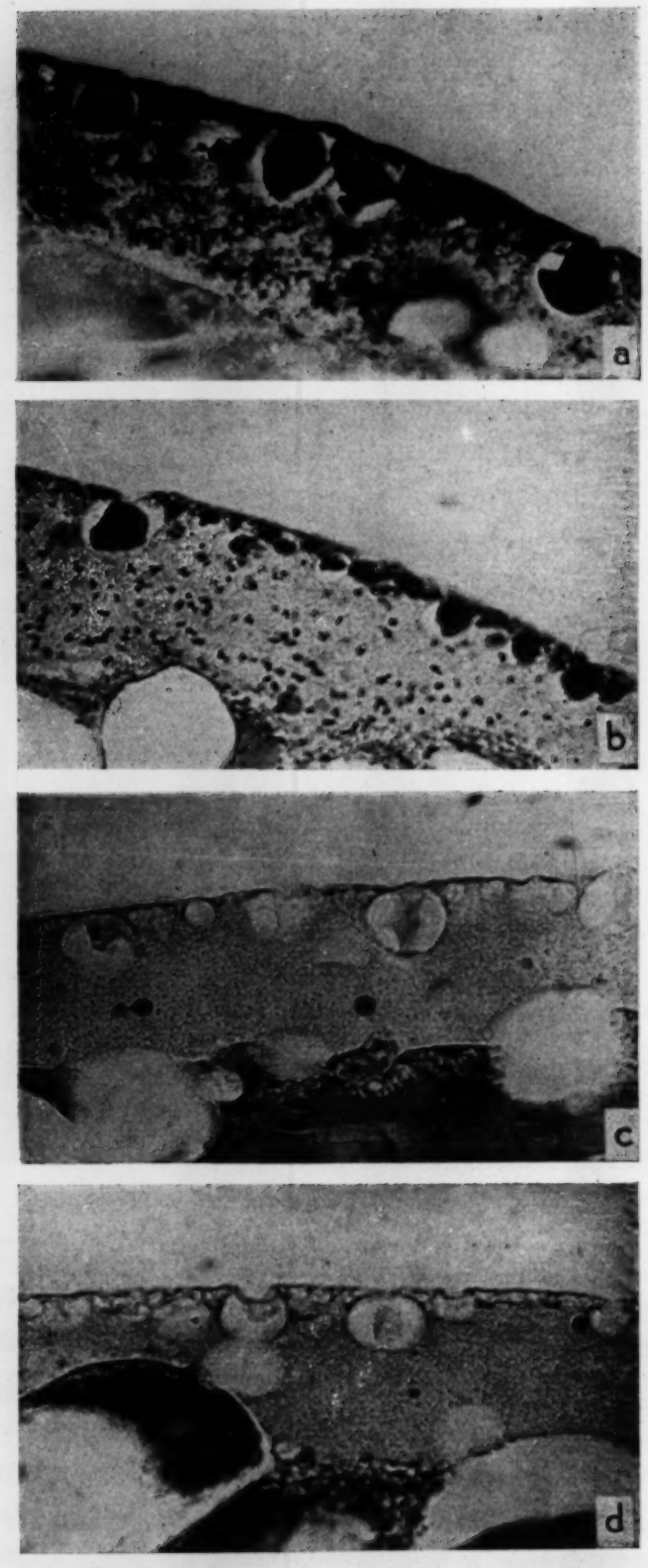


Fig. 10. Cytochemical staining of the alveoli of salmonid eggs. *a* and *b*, with the HOTCHKISS tests; *c* and *d*, with ninhydrin-SCHIFF's test. *a* and *c*, dog salmon egg; *b* and *d*, red salmon egg. ×450 (KUSA, 1954)

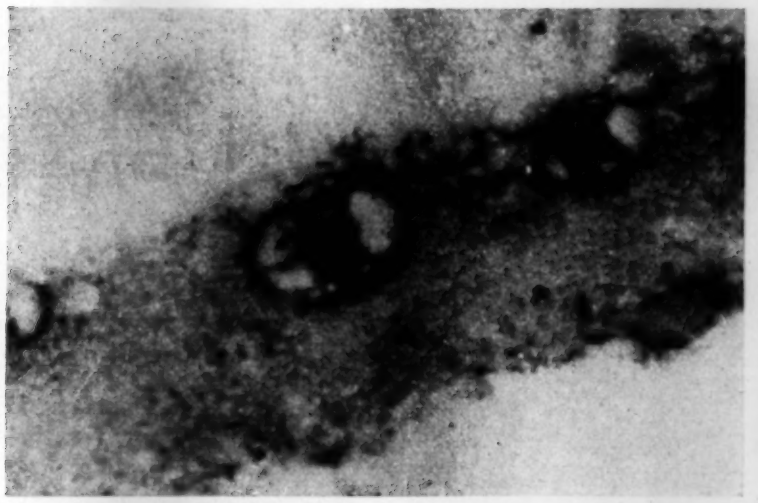


Fig. 11. A portion of peripheral cytoplasm of the unfertilized stickleback egg. The HOTCHKISS positive coagulum or fine network is seen within the alveoli. ×600 (KUSA, 1953 b)

with HEIDENHAIN's iron hematoxylin, are evently distributed throughout the ooplasm, but they are particularly concentrated at the boundary of each alveolus (Fig. 12 *a*). Though vital staining with Janus green B is not yet successful, these particles sharply stain with ALTMANN's acid fuchsin method or REGAUD's technique for mitochondria. Under the ordinary microscope, in fresh intact eggs, such fine particles are visible around each alveolus (Fig. 12 *b*). Even though not entirely convincing, it may be permitted to suggest that the surface of cortical alveolus is dense with the fine particles which resemble mitochondria. These particles are so firmly attached the alveolus that, on centrifugation, they move together with alveoli toward the heavy pole, as shown in Fig. 12 *d*. Probably the mitochondria-like particles constitute a regular element of the physical make-up of an alveolus. But their fate, subsequent to the alveolar breakdown, is not fully determined yet. In Fig. 12 *c* is shown an alveolus just in the process of breakdown, to which these fine particles still remain attached, except its opening.

In *Oryzias* eggs, T. YAMAMOTO (1951 a, b) has postulated the presence of lipoidal coating for each alveolus. This view is recently evidenced cytochemically by his student (AKETA, 1954). In the coating membrane of alveoli of the fish eggs here examined, however, contrary to all expectation, neither osmiophilic nor Ciaccio positive substance can be detected.

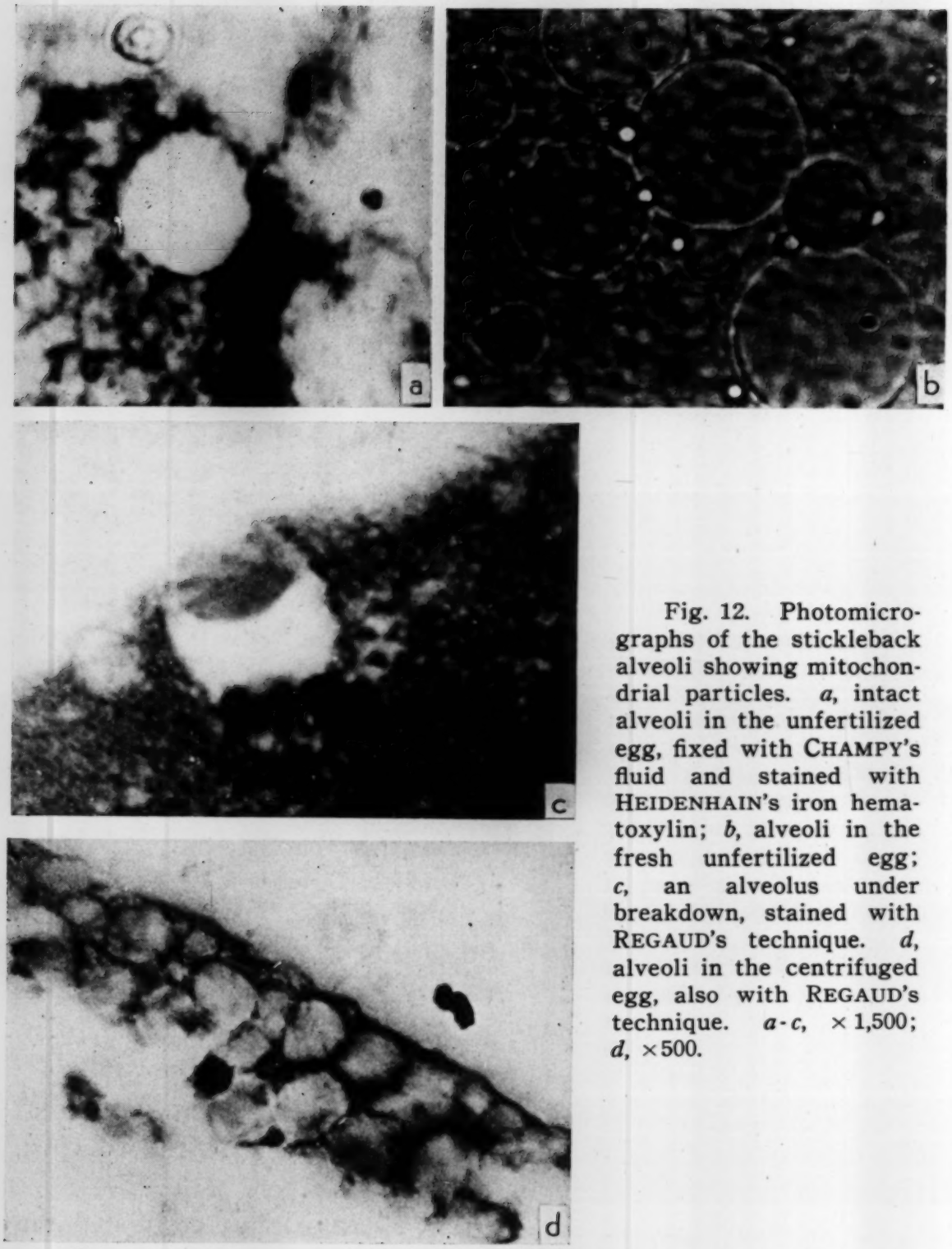


Fig. 12. Photomicrographs of the stickleback alveoli showing mitochondrial particles. *a*, intact alveoli in the unfertilized egg, fixed with CHAMPY's fluid and stained with HEIDENHAIN's iron hematoxylin; *b*, alveoli in the fresh unfertilized egg; *c*, an alveolus under breakdown, stained with REGAUD's technique. *d*, alveoli in the centrifuged egg, also with REGAUD's technique. *a*-*c*, ×1,500; *d*, ×500.

Cortical breakdown and initiation of development

Alveolar breakdown followed by the membrane elevation seems to be the first visible event on the egg surface in both fertilization and artificial activation. However, when unfertilized dog salmon eggs are

put in M/11 solution of $CaCl_2$ or $MgCl_2$, ooplasmic segregation takes place with little or no indication of alveolar breakdown, and the blastodisc is raised within a few hours at 10° C. Even hypertonic, but less than M/6, solutions of these salts are equally effective, while isotonic solutions of NaCl and KCl are of no effect (KUSA, 1953 a). As shown earlier (KUSA, 1950), the inseminated dog salmon eggs are not initiated into development, so long as they are kept in the RINGER solution, but incited anew, when immersed again in fresh water or the media, with which parthenogenetic activation can be induced. Moreover, it is of much interest that, with exposure to $CaCl_2$ or $MgCl_2$ solutions, cleavage occurs in almost all such RINGER-preserved inseminated eggs. Hence, it is tempting to suppose that the effect of bivalent cations (Ca and Mg) would be antagonistic to that of monovalent cations upon the developmental activity of the dog salmon eggs. As a matter of fact, M/11 solutions of either $CaCl_2$ or $MgCl_2$, if mixed with M/7.5 NaCl to at least three times its volume, becomes completely ineffective. But the eggs being placed back in fresh water after exposure to M/11 solution of $CaCl_2$ or $MgCl_2$, the alveoli readily break down and the perivitelline space is formed, unless the exposure is too long; beyond 8 hours (at 10° C.), breakdown is no longer possible. After all, the inhibitory action of these salts is reversible to some extent.

In the stickleback eggs, ten minutes after insemination or artificial stimulation, a rhythmic contraction begins, lasting about 25 minutes at the room temperature, and then the blastodisc comes to appear. On return to the RINGER solution followed by an extremely brief exposure to benzene vapor, they are activated parthenogenetically, just as *Oryzias* eggs (T. YAMAMOTO, 1950). After a little more prolonged exposure (30 to 45 seconds), the breakdown of alveoli takes place in the RINGER but apparently within a limited area of the egg surface. This is what is called "partial activation" with *Oryzias* eggs by T. YAMAMOTO (1949 b, 1951 b). With the partially activated eggs, he held that the conduction of the fertilization wave would in part be blocked. However, a close examination reveals that the breakdown still occurs in the stickleback alveoli even on the seemingly unaffected area and progresses in wave-like fashion as ever, even though it looks rather scatteringly to occur in a small number of alveoli. This observation may suggest that the breakdown of a

cortical alveolus is not the cause of the breakdown of neighboring alveoli and that the fertilization wave can propagate to some extent in seemingly unaffected regions in which most of alveoli have remained unchanged. If this suggestion is correct, the complete breakdown appears not always necessary for initiation of the developmental activity, at least in the eggs of dog salmon and sticklebacks.

General Consideration

Unfertilized eggs of some teleosteans are apparently characterized by the presence of alveolar structure in the cortex. In the eggs of *Gobio fluviatilis* and *Cyprinus carpio*, KONOPACKA (1935) first pointed out the content of mucoprotein in the "*gouttes claires*", which may be identified with the cortical alveoli of the present day, as judged from the figures appended to her paper. Subsequently to the writer's work (KUSA, 1953 b), ITO (1953) has proved in eggs of *Zacco* and *Plecoglossus* that the cortical alveoli selectively stain with MAYER's mucicarmine, a dye specific for mucous substance. More recently AKETA (1954) has demonstrated a polysaccharide substance in the alveoli of *Oryzias* eggs. Quite independent of these findings, THOMOPOULOS has arrived at a similar view on the chemical nature of alveolar content in the eggs of the teleosts, *Perca fluviatilis*, *Gasterosteus aculeatus* and *Ammdytes tobianus* (THOMOPOULOS, 1953 a, b, 1954). These data all agree in showing that the cortical alveoli contain some polysaccharide, probably esterified to sulfate, and chemically in common with the various teleostean eggs so far examined. But polysaccharide contained is neither glycogen nor hyaluronic acid. In any way, such high molecular polysaccharide substance within the alveoli appears to make up relatively heavy constituents of the fish eggs. Similar suggestive remark can be applied to the observations on the eggs of *Oryzias* (T. YAMAMOTO, 1944 b), *Plecoglossus* (ITO, 1954 b) and *Clupea* (YANAGIMACHI, unpublished data). Though alveoli give slightly positive reaction to ninhydrin-SCHIFF's reagent, it remains to be determined whether the proteins contained are linked to polysaccharides or freely dissolved within the alveoli. However, neither ribonucleic acid nor desoxyribonucleic acid is present. In *Cyprinus* egg, OJIMA (1943) held that the alveoli consist of fatty material. The similar view has been maintained by KAO and CHAMBERS (1954) with *Fundulus* egg, in which the "platelets," probably identical with the cortical alveoli, are stained with Sudan III. In these eggs, it seems to be highly probable that lipoid substance, if present, is contained in the coating membrane of alveoli, as in the *Oryzias* eggs (T. YAMAMOTO, 1951 a, b; AKETA, 1954). However, in any of the fish eggs here examined, no lipoid has yet been proved within the content of alveoli.

For the elevation of the egg membrane, a considerable amount of water must be taken up into the perivitelline space. In various eggs, the egg membrane is known to be permeable to water, inorganic salts and relatively simple organic substance, such as urea, glucose and amino acids, and also to some

dyes, but practically impermeable to such colloid substance as starch, egg alubumin and gum arabic (BODINE, 1928; ARMSTRONG, 1928; SVETLOV, 1929; BOGUCKI, 1930; GRAY, 1932). This holds true also for the present forms. In consequence, it seems that such high molecular polysaccharide substance of the alveoli must develop a colloid osmotic force of some strength beneath the egg membrane, when liberated in the perivitelline fluid. Thus, the egg activation is followed by water uptake, resulting in the membrane formation. This picture will confirm the view of BOGUCKI (1930) and T. YAMAMOTO (1939 b) as to the mechanism of the membrane elevation in fish eggs. According to T. YAMAMOTO (1951 a), when *Oryzias* eggs, with the cortical alveoli stained vitally with neutral red, are stimulated, colored aggregations of colloidal nature comes to appear on the surface, and gradually emerge into the perivitelline space. This observation seems of great importance in providing an indisputable evidence for the appearance of colloid substance beneath the egg membrane immediately after the alveolar breakdown.

Until recently, however, no minute structural change was examined with individual alveoli in the course of breakdown. In considering the data presented above, it becomes evident that the elimination of alveoli is not due to intracellular disintegration nor absorption, but to discharge of their content from the egg surface through aperture formed for each alveolus (KUSA, 1953 b). Immediately after the writer's work was published, such a breakdown process of individual alveoli has been well corroborated by ITO (1954 a) with the eggs of *Plecoglossus* and *Zacco*. In all probability, upon egg activation, a certain change or changes must take place in the state of molecular aggregation in either content or coating membrane, or both of each alveolus, since, preceding the breakdown, the optical property of alveoli is greatly changed. T. YAMAMOTO (1951 a, b) suggested that the breakdown of alveoli is intimately associated with activation of some esterases responsible for dissolution of lipoid phase on the alveolar wall as well as on the egg surface. If this is the case, such enzymatic action would operate on the aperture formation on each alveolus. Perhaps on account of his unsuccessful attempt to find out the alveolar structure, AOKI unfortunately claimed that the perivitelline space is formed in the dog salmon egg by peptization of extracellular colloid aggregates believed to pre-exist beneath the egg membrane and a remarkable osmosis resulted therefrom (AOKI, 1942). But in the presence of the above mentioned irrefutable evidence, this postulate by AOKI is now quite untenable. However, in some fish eggs, the separation of egg membrane is accomplished by the corresponding shrinkage of the egg proper, as shown in the eggs of *Fundulus* (KAGAN, 1935; KAO and CHAMBERS, 1954) and *Clupea* (KANOH, 1953). In fact, the stickleback eggs, when activated, slightly shrink all over the surface, accordingly, the egg membrane being separated chiefly by extending the membrane itself, but in part by shrinking the egg proper. In contrast, in salmonid eggs, such shrinking cannot be observed on activation (AOKI, 1939). But in either cases, there is no room for doubt that development of colloid osmotic force in the perivitelline fluid makes the whole egg rather turgid, and this in turn exerts a strong hydrostatic pressure on the

egg proper, as is recently shown in *Fundulus* egg by KAO and CHAMBERS (1954).

The surface of each alveolus in the stickleback egg is dense with the fine mitochondria-like particles. These fine particles may be highly comparable with T. YAMAMOTO's "cortical granules A" in *Oryzias* egg, at least in structural respect. In *Oryzias* eggs, the cortical granules A are about 0.5 μ in diameter and also closely attached to the surface of alveoli; they are believed to be bearers of some esterases, which, at the time of egg activation, dissolve lipoid substance in both alveolar wall and egg surface (T. YAMAMOTO, 1951a, b). In view of the recent development in physiology of mitochondria, it would be conceivably that these mitochondrial particles play some important role in function of the alveoli or even of the whole egg.

Under certain experimental conditions, some unfertilized teleostean eggs begin to develop, with the alveolar breakdown being incomplete. Beside the writer's work on the egg of dog salmon (KUSA, 1953a), similar facts have been observed with the eggs of *Clupea* (KANOH and YANAGIMACHI, 1953), *Tribolodon* (KANOH and ITO, 1953), *Perca* (THOMOPOULOS, 1953a), *Gasterosteus* (THOMOPOULOS, 1953b) and *Salmo* (DEVILLERS, THOMOPOULOS and COLAS, 1953). Therefore, it may be concluded that the alveolar breakdown is not essentially a prerequisite to initiation of development, but appears to be no more than an accessory event to it, so that under usual conditions it merely occurs as if it is seemingly indispensable. Consequently, the membrane elevation caused by discharge of the alveolar content into the perivitelline space is probably not essential for the development. This may account, at least in part, for the fact that, under certain experimental conditions, some animal eggs develop without membrane elevation.

So-called "Janus green granules" (MOTOMURA, 1936, 1941) or "cortical granules" (MOSER, 1939a; RUNNSTRÖM *et al.*, 1946) in sea urchin eggs bear striking resemblance in many respects. These granules are relatively heavy (MONNÉ and HÅRDE, 1951), semifluid (MOTOMURA, 1941; CHENEY, 1951), and on either fertilization or artificial activation, optically changing (ENDO, 1952), they break down in a wave-like fashion from one pole to the other (MOSER, 1939a, b; ENDO, 1952: SUGIYAMA, 1953a, b; ALLEN, 1954). Moreover, under certain conditions, they remain unchanged even after development has begun (MOTOMURA, 1952a; MONNÉ and WICKLUND, 1947; IIDA, 1947). The cortical granules of similar nature are also present in the egg of many other animals.[5] On the chemical side, MONNÉ and HÅRDE (1951) have advanced the view that the cortical granules of some sea urchin eggs are composed in the main of sulfated polysaccharide. Similar view can be drawn from the observation on the egg of *Nereis* (*cf.* COSTELLO, 1949) and *Tylorrhynchus* (T. YAMAMOTO, 1952), and the test-cells of *Cynthia* eggs, these cells being also discharged

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[5] E. g., the polychaete worm, *Nereis limbata* (LILLIE, 1911; COSTELLO 1949) and *Tylorrhynchus heterochaetus* (T. YAMAMOTO, 1952), the crinoid, *Comanthus japonicus* (DAN, J. C. and K. DAN, 1941), the starfish, *Asterina pectinifera* (MOTOMURA, 1941), the frog, *Rana nigromaculata* (MOTOMURA, 1952b) and the lamprey, *Lampetra planeri* (T. YAMAMOTO, 1944c, 1947).

from the egg surface on fertilization (HIRAI, 1941, 1949). In several marine eggs, according to KELLY (1951), not only the jelly coat but also certain cortical structures show a metachromatic shade characteristic of polysaccharide with toluidine blue. In consequence, rupture and discharge of these cortical structures or the likes, all with such polysaccharide substance contained, appear to be of rather general occurrence in both fertilization and artificial activation of the egg. Thus it leads us to venture the hypothesis that activation may take place on the basis of a certain general plan, perhaps common to the eggs of various animal species.

In the kinase theory of RUNNSTRÖM (1949), the activation of eggs begins with release of a kinase from an inhibitory substance, probably a mucopolysaccharide of heparin-like nature. This theory is, so to speak, a modern reformulation of the early "inhibition theory" by F. R. LILLIE (1911). But, as for the release of kinase from inhibitor, no conclusive evidence was presented in structural aspect. In the goldfish, TCHOU and CHEN (1936) postulated that the content of cortical alveoli is a certain waste metabolite, storage of which gives rise to autointoxication of the ooplasm, so that "*épuration*" or elimination of it is necessary for the egg activation. In the light of chemical nature of mucopolysaccharide within the alveoli, their concept of "*épuration*" may be said to be associated largely with the kinase theory by RUNNSTRÖM. In fact, heparin or other related polysaccharides are known to exert inhibitory effects upon various cellular activities (HEILBRUNN, 1952). MONNÉ and his coworkers stated that, on fertilization of sea urchin eggs, hydration is markedly increased in the cortex by action of acid polysaccharide contained in both cortical granules of the egg and acrosome of the spermatozoön introduced (MONNÉ and SLATTERBACK, 1950; MONNÉ and HÅRDE, 1951). At the time of egg activation, according to the suggestion by RUNNSTRÖM (1951), certain submicroscopic fibrils will be re-arranged within ooplasm as a consequence of breaking up their carbohydrate cements. These statement and suggestion may point out the importance of carbohydrate, especially of polysaccharide, in relation to which some basic submicroscopic change or changes take place in the cortical layer, and result in the visible structural changes thereafter. In conclusion, it is of no doubt that the polysaccharide substance within the cortical alveoli plays a certain essential rôle in the structural change in the cortex on fertilization and artificial activation of fish eggs.

CONCLUSION AND SUMMARY

In the eggs of two stickleback and four salmonid species, the cortical layer is dense with so-called "cortical alveoli," except the animal polar region. These alveoli are varying in size, highly refractive and relatively heavy within ooplasm. Their content is chiefly composed of mucopolysaccharide of heparin nature, with protein reactions being negative or only slightly positive, but neither lipoid nor nucleic acid is presented within. When the eggs are inseminated or

artificially stimulated, the whole alveolar structure breaks down in a wave-like fashion from one pole to the other. However, strictly speaking, the alveoli break down more or less independently of one another and even at random, so that a wave-like breakdown, conveniently so called, should merely be taken as a statistic or collective manifestation of individual process.

Upon breakdown, each alveolus forms an aperture, through which it is emptied of the content beneath the egg membrane. The high molecular polysaccharide thus liberated develops a remarkable colloid osmotic force, which gives rise to water uptake and results in formation of perivitelline space, separating the egg membrane from the egg proper. To the surface of each alveolus in the stickleback eggs are closely attached the finner particles highly comparable in character with mitochondria. These particles may be associated either functionally or structurally, with a delicate envelope, which becomes visible at the boundary of the alveoli isolated in the RINGER solution, when such alveoli are ruptured spontaneously or by puncturing with a glass needle.

In solutions of $CaCl_2$ or $MgCl_2$, the blastodisc is raised in the unfertilized dog salmon eggs, with little or no indication of alveolar breakdown. In the stickleback eggs, when placed back in the RINGER after a brief exposure to benzene vapor, the alveolar breakdown occurs only within a limited area most intensely affected; notwithstanding, the blastodisc formation takes place as in usually stimulated eggs. Moreover, a close examination reveals that, even on the seemingly unaffected area of such surface, the breakdown occurs still in a wave-like fashion, though it looks scatteringly to occur in a small number of alveoli. All these facts may suggest that the alveolar breakdown appears not to be a prerequisite to initiation of the egg activation, and also that an ooplasmic excitation-conduction (YAMAMOTO's "fertilization wave") progresses to some extent in seemingly unaffected regions in which most of the alveoli have remained unchanged.

In conclusion, teleostean cortical alveoli may have many characteristic traits, in both structural and chemical aspects, in common with surface granules or inclusions found in many invertebrate eggs, especially with cortical granules of sea urchin eggs. The egg activation would be based on a certain general plan, probably common to various animal eggs.

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STUDIES ON THE FORMATION OF FISH EGGS

VIII. THE FATE OF THE YOLK VESICLE IN THE OOCYTE OF THE SMELT, *HYPOMESUS JAPONICUS*, DURING VITELLOGENESIS*

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As was previously pointed out, the yolk vesicles of fish oocytes are characterized by the predominant polysaccharide contents and they originate from argentophilic bodies (K. YAMAMOTO 1955 a, b, 1956 c). The preceding work concerning the fate of the yolk vesicle of *Clupea pallasii* led to the conclusion that the vesicles contain mucoid throughout the course of the vitellogenesis and they appear to give rise to the cortical alveoli after migrating towards the periphery of the ooplasm (K. YAMAMOTO 1956 d). Since these findings seem to present an important clue as to the vitellogenesis of fish eggs, more evidence bearing upon the problem has been collected, using the oocytes of *Hypomesus japonicus*.

Before proceeding further, the author would like to express here his cordial thanks to Professor TOHRU UCHIDA for his kind revision of the manuscript, and to Professor SAJIRO MAKINO for helpful advices given to the author.

MATERIAL AND METHOD

The oocytes of the smelt, *Hypomesus japonicus*, were obtained in a similar way as that described in the former paper (K. YAMAMOTO 1955 b). Ovaries were fixed with BOUIN-ALLEN's mixture, formol-alcohol fluid and REGAUD's solution. Sections, 10 to 12 μ in thickness, were prepared by the usual paraffin method. For the demonstration

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of polysaccharides in the oocytes the P.A.S. method (cited from GLICK 1949) and BAUER's test (cited from LISON 1953) were adopted. Moreover, the P.A.S.-positive substances were examined by the saliva test for glycogen (cited from ICHIKAWA 1953), the metachromatic staining with toluidine blue for sulfated mucopolysaccharides (cited from GLICK 1949) and by HIGHMAN's method for the determination of the relative acidity of mucoprotein (cited from ICHIKAWA 1953). Details concerning the practice of these cytochemical techniques may be found in the preceding paper (K. YAMAMOTO 1956 d).

OBSERVATIONS

1. The fate of the yolk vesicle during vitellogenesis

The fate of the yolk vesicles of the smelt can be easily traced because of their strong P.A.S. reaction. At the beginning of their formation yolk vesicles appear as minute granules arranged near the periphery of the ooplasm in a single row (Fig. 1 *a*). The vesicles then increase rapidly in size, but slowly in number. Along with the formation of the vesicle, the yolk globules appear independently in the extravesicular cytoplasm. In the early stage, the globules are distributed sparsely in the outermost part of the ooplasm, and then rapidly increase in number to form a thick layer. Each globule is minute in size and exhibits a very faint P.A.S. reaction (Fig. 2). Hereafter, both the vesicles and globules seem to be formed centripetally till the ooplasm comes to be thoroughly filled with them at the end of the yolk vesicle stage (Figs. 3 and 4 *a*). At the primary yolk stage,* the yolk vesicles of large size, whose dimensions ranged about 20 to 36 μ in long axis, are located in the peripheral part of the ooplasm and the small yolk globules chiefly in the central part. In addition to the two kinds of vitelline particles, there could be found a third kind of vitelline elements distributed at random in the ooplasm. In BOUIN and P.A.S. preparations they appeared as empty spaces, in unstained CHAMPY preparations as blackened particles and in frozen sections stained by Scharlach *R* as scarlet droplets (Figs. 5 *a* and 5 *d*). Therefore, the last-mentioned vitelline elements may be regarded with certainty as fatty droplets. During the yolk formation stages more advanced than the previous stage, the yolk globules and

* Cf. K. YAMAMOTO 1956 a, p. 364.

the fatty droplets become more and more numerous with the growth of oocytes, to occupy large part of the ooplasm. On the other hand, the yolk vesicles change their distribution without changing their size and number during the period. Being gradually shifted outwards, the vesicles are found arranged in the periphery of the ooplasm, roughly forming a row (Fig. 6). In Fig. 7 is shown a section of ripe eggs. In the cortical layer the cortical alveoli are found embedded, measuring 16 to 40 μ in long axis. The coagulum found inside the alveoli reacted positively on the P.A.S. test and showed a metachromasy with toluidine blue. Judging from the size, location and polysaccharide contents of the alveoli, it is reasonable to surmise that the yolk vesicles give rise to the cortical alveoli as they do in the oocytes of the herring (K. YAMAMOTO 1956 d).

2. On the nature of the polysaccharide substances occurring in the yolk vesicles

Results obtained from cytochemical tests are summarized in Table 1. It is worth noting that the yolk vesicles show no metachromatic nature in the oocytes of the yolk vesicle stage, while in more advanced stages they become metachromatic. At the yolk vesicle stage, the contents of the yolk vesicles were positive for the P.A.S. and BAUER's tests. Saliva was inactive to the P.A.S.-positive substances. Moreover, the vesicles were neither stained metachromatically with toluidine blue nor with methylene blue in acid solution, regardless whether or not the preparations were pre-treated with chromic acid (Fig. 1 *b*). Even in case toluidine blue metachromasy was not found in the vesicles treated with chromic acid, by taking

Table 1. Staining of mucopolysaccharide substances in the yolk vesicles by several cytochemical techniques

	P.A.S. reaction	BAUER's reaction	Metachromasia before chromic acid treatment	HIGHMAN's test before chromic acid treatment	Metachromasia after chromic acid treatment
Yolk vesicle stage	###	##	–	pH 3 – 4 – 5 – 6 –	–
After yolk vesicle stage	##	##	###	pH 3 ### 4 ### 5 ### 6 ###	–

Intensities of reactions are indicated by marks – to ###.

in consideration the results of other tests, such as P.A.S. and HIGHMAN's reaction, which gave the positive evidences for the presence of neutral mucopolysaccharides, it seems justifiable to think that the mucopolysaccharides occurring in the vesicles of this stage are regarded as neutral mucopolysaccharides (mucoids).

Even after the yolk vesicle stage, the presence of mucopolysaccharides was also demonstrated in the vesicles by the cytochemical tests as above. In contrast to the vesicles in the oocytes of the yolk vesicle stage, however, the vesicles in more advanced stages gave a brilliant toluidine blue metachromasy. By staining 0.1 per cent solution of toluidine blue in 1 per cent alcohol, the vesicles were deeply stained purple. The coloration remained almost unchanged even after being mounted with canada balsam (Fig. 5 *b*). Further, the metachromatic vesicles were also stained deeply with methylene blue in acid solutions (Fig. 5 *c*), although the coloration by methylene blue was very labile, and it nearly disappeared when preparations were dehydrated in alcohol and xylol. Following the treatment with chromic acid, the metachromatic nature of the vesicles was lost completely. The vesicles, when pre-treated with 10 per cent chromic acid for 4 hours followed by toluidine blue, showed no signs of metachromasy (Fig. 4 *b*). A similar negative result was obtained with methylene blue staining following chromic acid treatment. From the above findings it is clear that the polysaccharide substances abundant in the vesicles change their nature in the course of vitellogenesis.

According to the recent study of BRADEN (1955), only the sites where mucoids associated with acid mucopolysaccharides are present, exhibit both a strong P.A.S. reaction and deep toluidine blue metachromasy. Therefore, it is most reasonable to regard the polysaccharide substances occurring in the vesicles of the advanced stage as mucoids associated with acid mucopolysaccharides.

DISCUSSION

From the findings described above it seems beyond doubt that the fate of the yolk vesicle of the smelt is quite similar to that of the herring (K. YAMAMOTO 1956 d). The vesicles of the smelt exhibited always a strong P.A.S. reaction throughout vitellogenesis. On moving towards the periphery, they were arranged in a row and

appeared to develop into the cortical alveoli which play an important role at fertilization. On the other hand, the yolk globules and the fatty droplets were accumulated in the cytoplasm, independently of the yolk vesicles. The globules were always negative or very slightly positive to P.A.S., and the droplets are mainly composed of lipids. There remains little doubt that these vitelline particles are supplied as nutritive material for the later development of fish eggs. In addition to these facts, a previous study (K. YAMAMOTO 1955 b) showed that the yolk vesicles appear to have their origin in argentophilic bodies, while the yolk globules are probably derived from mitochondria. Therefore, the yolk vesicles differ from the other yolky substances in their origin and fate in vitellogenesis and in the role in development.

Recently, T. S. YAMAMOTO (1955), who studied the oogenesis of *Oryzias*, made clear that the vacuole-like bodies previously reported by AKETA (1954) contain acid mucopolysaccharides and strictly correspond to the yolk vesicle of GUTHRIE (1928). Although his material is vastly different in the mode of yolk formation from herring and smelt oocytes, he has come to the same conclusion, so far as the fate of the yolk vesicle is concerned, *i.e.*, that the yolk vesicles give rise to the cortical alveoli.

According to the nature of polysaccharide substances occurring in yolk vesicles, fish eggs can be classified into three types. The yolk vesicles in the oocytes of the first type contain mucoids alone throughout vitellogenesis. Herring eggs represent this type (K. YAMAMOTO 1956 d), and flounder eggs seem also to be included in this type (K. YAMAMOTO 1956 b, c). In the eggs of the second type, mucoids present in young vesicles change in nature in the course of vitellogenesis, and fully grown vesicles contain mucoids associated with acid mucopolysaccharides. As has been demonstrated in this study, smelt eggs belong to the second type. Besides the smelt eggs, salmon eggs appear to be included in this type. According to KUSA (1954), the contents of the cortical alveoli of salmon eggs are composed of mucoprotein containing polysaccharide esters with sulfuric acid. Except HIGHMAN's test which has not been tried by him, cytochemical results obtained in the salmon fit in completely with those in the smelt. Therefore, the polysaccharides occurring in the alveoli of salmon eggs may also be regarded as mucoids associated with acid

mucopolysaccharides. On the other hand, the present writer has observed that the yolk vesicles found in young oocytes of the salmon, *Oncorhynchus keta*, react positively to the P.A.S. test, but negatively to HIGHMAN's test and LISON's metachromasia test (unpublished). Therefore, the mucoidal substances detected in salmon vesicles are believed to be changeable into mucoids associated with acid mucopolysaccharides in the course of vitellogenesis. The eggs of the third type has recently been reported by T. S. YAMAMOTO (1955). That is *Oryzias* eggs, of which the yolk vesicles contain acid mucopolysaccharides alone throughout the vitellogenesis.

Regarding the embryological significance attributable to the difference of polysaccharide contents of the vesicles, the author has not yet reached any definite idea. A possible significance may be found in the difference of spawning habit of the fishes, *i.e.*, the eggs belonging to the first type are of marine fishes, those belonging to the second type are of anadromous fishes, and those of the third type are of a fresh water fish.

SUMMARY

In the oocytes of the smelt, there can be found three kinds of vitelline particles, *i.e.*, yolk vesicles, yolk globules and fatty droplets. Of these particles the yolk vesicles seem to be quite different from others both in origin and function. The vesicles seemingly derived from argentophilic bodies are formed as the first vitelline particles and develop subsequently into the cortical alveoli. The predominant components of the vesicles consist of polysaccharides. In the course of vitellogenesis the polysaccharide substances in the vesicles change from neutral mucopolysaccharides into mucoids associated with acid mucopolysaccharides.

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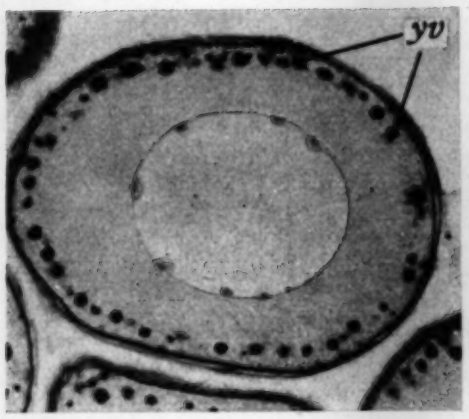
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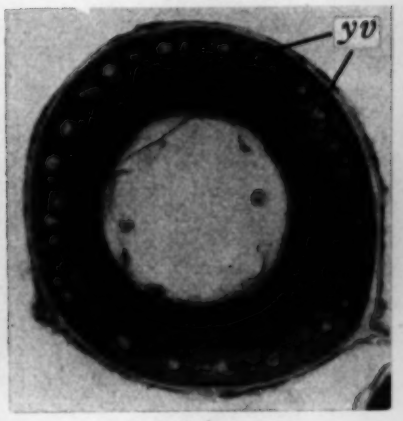
Explanation of Plate 2

All figures are photomicrographs from sections of smelt oocytes.
Fig. 1 *a*. Bouin-Allen and P.A.S. preparation.
Fig. 1 *b*. Bouin-Allen and methylene blue (pH 6) preparation.
Fig. 2. Regaud and P.A.S. preparation.
Figs. 3 and 4 *a*. Bouin-Allen and P.A.S. preparations.
Fig. 4 *b*. Bouin-Allen and toluidine blue preparation with chromic acid treatment.
Fig. 5 *a*. Bouin-Allen and P.A.S. preparation.
Fig. 5 *b*. Bouin-Allen and toluidine blue preparation.
Fig. 5 *c*. Bouin-Allen and methylene blue (pH 3) preparation.
Fig. 5 *d*. Unstained Champy preparation.
Fig. 6. Regaud and toluidine blue preparation.
Fig. 7. Champy and toluidine blue preparation.

yv Yolk vesicle, *yg* Yolk globule, *fd* Fatty droplet, *ca* Cortical alveolus, *cl* Cortical layer.



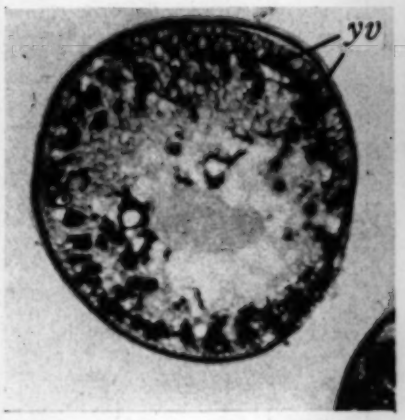
1 *a*



1 *b*



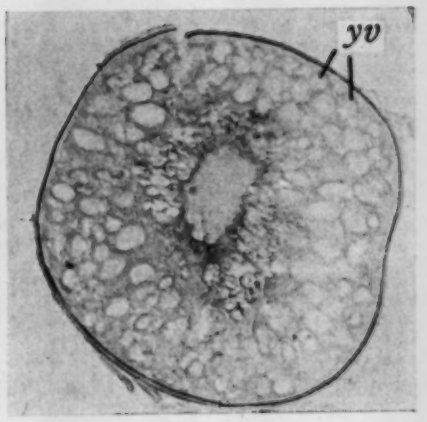
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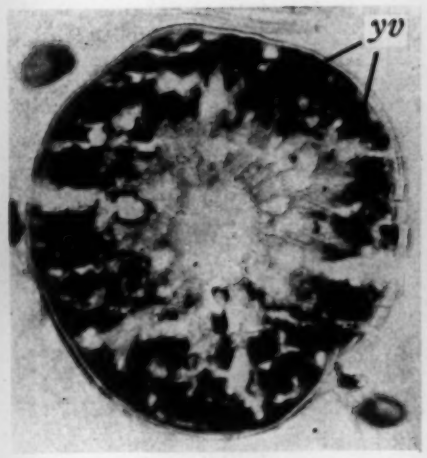
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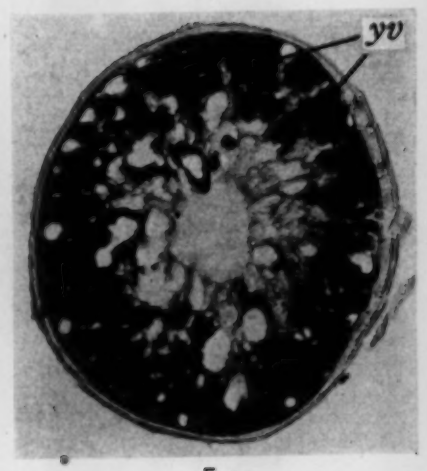
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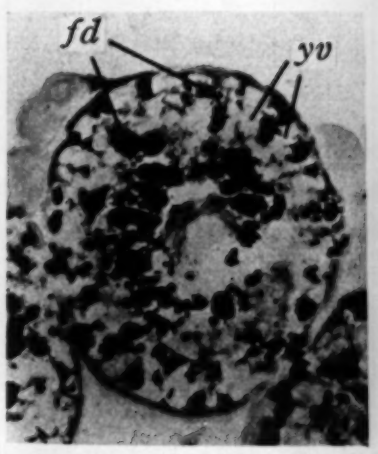
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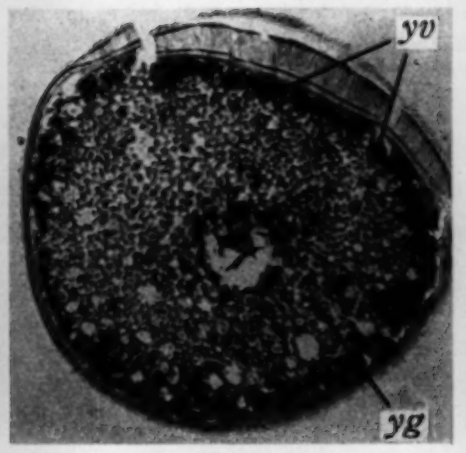
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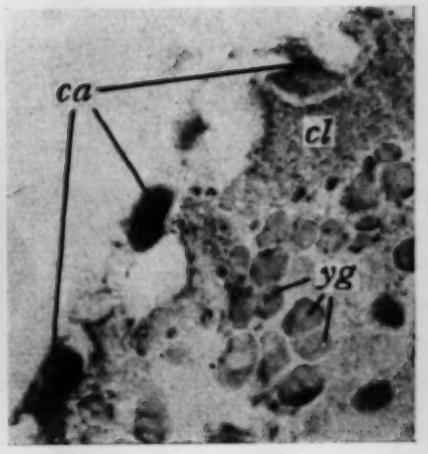
5 *c*



5 *d*



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PHYSIOLOGICAL STUDIES ON RE-FERTILIZATION OF THE SEA URCHIN EGG*

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INTRODUCTION

It is well known that fertilized eggs of the sea urchin cannot be re-fertilized even if the fertilization membrane has been removed in normal sea water. This fact suggests that there is some sort of sperm block on the surface of the fertilized egg. Recently, it has been found that when activated or fertilized eggs were washed with Ca-Mg-free medium after removal of the fertilization membrane, they again become penetrable to fresh spermatozoa (ISHIDA and NAKANO 1950, SUGIYAMA 1951). In view of this finding, the sperm block on the egg surface appears to be broken down by washing with Ca-Mg-free medium. The possibility was considered that the substance of the sperm block might be contained in the supernatant from such washings. The earlier results, given in a preliminary note (NAKANO 1952, 1953), showed that denuded eggs give off a substance into Ca-Mg-free medium which is capable of preventing fertilization. In this connection, it was pointed out that the fertilized egg of the sea urchin is surrounded by a hyaline layer which dissolves in a Ca-Mg-free medium (MOORE 1930). Furthermore, both the hyaline layer and the sperm block were found to be affected if the eggs were activated in the presence of polyspermy-inducers (NAKANO 1954). Hence, it was suggested that the sperm block on the egg surface is closely associated with the hyaline layer and that the substance obtained may be referred to as "hyaline layer sub-

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stance." The present paper is concerned with the characteristics of the hyaline layer particularly with respect to its chemical nature and relationship to the sperm block which prevents re-fertilization. Additionally, the mechanism of the formation of the sperm block is discussed. The sperm block on the surface of the fertilized egg answers the question of the resistance to superimposed fertilization; however, there is evidence which suggests that this block is connected with the natural mechanism for the prevention of polyspermy. Recently HAGSTRÖM and HAGSTRÖM (1954 a) confirmed that re-fertilization is possible by treatment with Ca-Mg-free media in the trypsin-treated egg. ALLEN and HAGSTRÖM (1955 a, b) advanced the hypothesis that the hyaline layer plays an important role in the prevention of polyspermy.

The block to polyspermy has caused interest among many previous investigators. FOL (1879) considered that the fertilization membrane prevented the entrance of supernumerary spermatozoa and constituted the block to polyspermy. This view is not accepted any longer, because the formation of the fertilization membrane appears much too slow to prevent polyspermy. JUST (1919) stated that the mechanism of monospermy is attributable to a so-called "wave of negativity" that sweeps over the egg surface preceding the membrane elevation. Its nature, however, has not yet been elucidated. More recently ROTHSCHILD and SWANN (1949) pointed out that the propagation of the wave must not necessarily be very rapid. As a criterion of the propagation of the wave they utilized the color change of the egg surface which can be observed with dark ground illumination. The conduction time of this change was found to be of the order of 20 seconds at 18° C. in *Psammechinus* eggs. They assumed that this visible change may constitute the block to polyspermy. In later works, ROTHSCHILD (ROTHSCHILD and SWANN 1952, ROTHSCHILD 1953) suggested that the block to polyspermy is diphasic, there being a fast component which covers the egg in less than 2 seconds and a slower component which makes the egg totally impermeable to spermatozoa. He stated that the change in cortical structure is clearly a reflection of this slow component. For further references on this subject, readers must refer to the recent review by ROTHSCHILD (1954).

As far as the chemical nature of the sperm block is concerned, there is at present comparatively little information. RUNNSTRÖM *et al.* (1944, 1945, 1946) found that a substance obtained by methanol extraction of the sea urchin sperm is able to inhibit fertilization without immobilizing the spermatozoa. This agent was designated "sperm lysin" and considered to be unsaturated fatty acid. RUNNSTRÖM suggested that the sperm lysin is released at the egg surface upon the attachment of one spermatozoon, and perhaps plays an important role in preventing the attachment of supernumerary spermatozoa. However, whether it is available under physiological conditions does not appear to have been established. Although the experiments to be reported here are limited to the sperm block which prevents re-fertilization, they constitute an attempt to correlate the chemical and physiological aspects of the sperm block.

MATERIALS AND METHODS

The materials used in these experiments belong to the two sea urchin species, *Hemicentrotus pulcherrimus* and *Pseudocentrotus depressus*. The eggs of *Hemicentrotus* were used for the majority of the experiments since the fertilization membrane can be easily removed and the clear protoplasm renders mitotic figures easily visible in the living eggs. The eggs were obtained by inducing shedding into filtered sea water. This was accomplished by introducing a few drops of isotonic KCl into the oral opening. The eggs, obtained in this way, were washed with filtered sea water and only eggs from lots showing 95 to 100 per cent membrane elevation were used. Sperm were obtained by removal of the testes from the animal. Dry sperm, shed from the isolated testes, was taken as a standard and various concentrations of sperm were made from this by dilution. In every case, only freshly suspended spermatozoa were used. A 10^{-2} sperm suspension of *Hemicentrotus* contains approximately 2×10^8 spermatozoa per ml.

The general procedures used in microscopical experiments were as follows. The eggs were previously deprived of their jelly coats by washing with acid sea water (pH 4.2). The first insemination was performed in the usual manner. Immediately after fertilization,

the fertilization membrane was removed by repeatedly sucking and blowing the eggs with a narrow pipette. In the first series of experiments, this procedure was carried out in a molar urea solution. The denuded eggs were then exposed to the hyaline layer substance. After immersion for 10 to 20 minutes, the eggs were inseminated again.

In another series of experiments, fertilized eggs were put into the solutions of polyspermy-inducers and removal of the fertilization membrane was performed in it. Thereafter the denuded eggs were inseminated again. In some cases, superimposed insemination was performed without removal of the membrane. The eggs were allowed to develop to the first cleavage and the percentages of unfertilized, monospermic and polyspermic eggs were counted. The re-fertilized eggs showed abnormal mitotic figures and cleavages characteristic of polyspermic eggs. In all the experiments, parallel controls were run without treatment by the test solution to check the incidence of re-fertilization resulting from injury incurred during the handling of the eggs. The experiments were carried out at room temperature (about 15° C. for *Hemicentrotus* and about 20° C. for *Pseudocentrotus*).

Experiments and Results

Preparation and properties of the hyaline layer substance

The hyaline layer substance was prepared from the eggs of *Hemicentrotus*. The eggs were fertilized and the fertilization membrane was removed immediately after its elevation by filtering with bolting silk (Lindahl and Lundin 1948). This procedure removes the fertilization membranes from more than 90 per cent of the eggs without harming them. The denuded eggs adhere easily to each other and they often burst when washed in sea water. This inconvenience was avoided to a considerable extent by treating the egg suspension with sea water acidified to a final pH of about 7. It was found that this treatment prevents the adhering of the eggs without disturbing the formation of the hyaline layer substance. The denuded eggs were placed therein for 15 minutes, in rather dilute population, then concentrated by sedimentation under gravity. After

repeated washing, the eggs were treated with a molar urea solution, in which the hyaline layer was completely dissolved without injury to the eggs. In some experiments, the eggs were treated with 5/9 M NaCl instead of the urea solution. The egg suspension was centrifuged after 5 minutes and the supernatant solution decanted off and filtered. To this solution, ammonium sulphate was added to 50 per cent saturation. After standing overnight, the precipitate was sharply centrifuged down, dissolved in water and dialyzed against running tap water and finally distilled water. Then the solution was precipitated again with ammonium sulphate. In this state, the gelatinous substance obtained may be preserved in the cold for a considerable period. When necessary, the precipitate was dialyzed again and frozen dried. In typical experiments, 8.0 mg. of the hyaline layer substance was obtained from 10 ml. of concentrated denuded eggs.

The aqueous solution of the hyaline layer substance shows high viscosity and tends to become insoluble in water after drying. The hyaline layer substance gives positive MILLON's, Biuret and MOLISCH's tests and contains 8.5 per cent total Kjeldahl nitrogen. The presence of fucose was demonstrated by paper partition chromatography on a hydrolyzed preparation (NAKANO and OHASHI 1954). Quantitative estimations of sugar were carried out according to the modified method of DISCHE and POPPER (1926) and gave an approximate amount of 7.2 per cent. These results indicate that the hyaline layer substance contains mucopolysaccharides. Furthermore, the observations that the hyaline layer substance is well precipitated with Janus green, toluidine blue or trypaflavine constitute an additional evidence for this view.

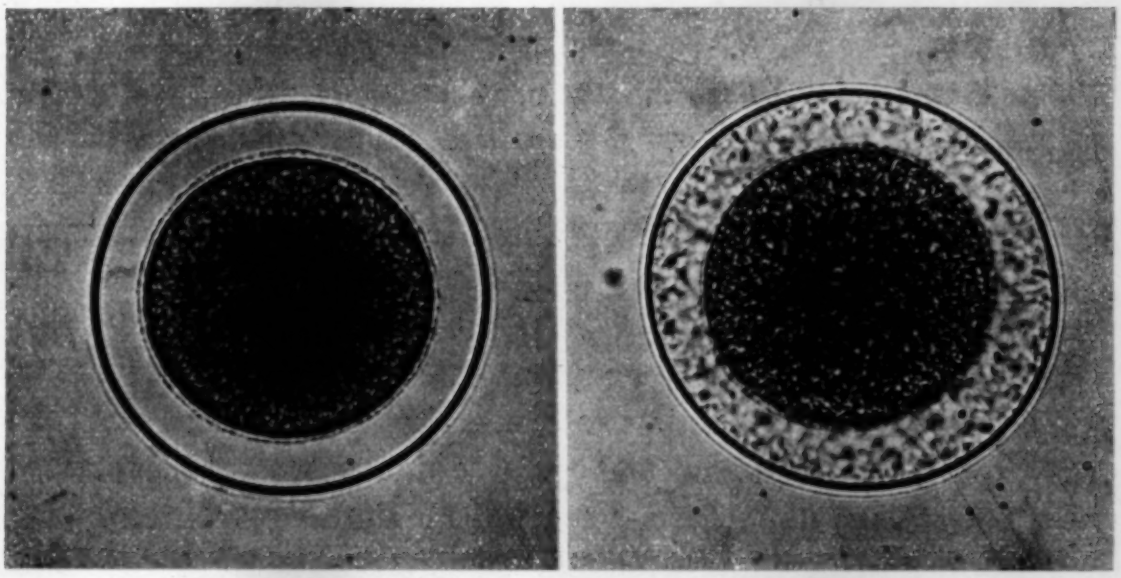
Preliminary experiments showed that the hyaline layer substance is nearly homogeneous in electrophoresis at pH 7.0 (phosphate buffer). These results do not, however, exclude the possibility that the hyaline layer contains several substances which are firmly associated or have the same electrophoretic properties. There is still a possibility that the hyaline layer substance contains a membrane-like substance in addition to the main component. Besides, ALLEN and HAGSTRÖM, (1955 b) suggested that the hyaline layer may have a dual origin and composition.

The hyaline layer substance was only slightly soluble in sea water and, therefore, its inhibiting action was weak as described below. However, it was found that if the aqueous solution of the hyaline layer substance was heated at 95° C. for 30 minutes, it became soluble in sea water and showed activity in preventing fertilization to a considerable extent. The hyaline layer substance could even be subjected to heating at 120° C. in an autoclave for 10 to 30 minutes to have its inhibitory effect on fertilization only enhanced. Consequently, most physiological studies were carried out on the autoclaved preparation. It is noteworthy that there was no precipitate in these preparations. Since calcium is the predominating cation of the hyaline layer, this suggests that the calcium bridge may be broken by these treatment. For further analysis, the hyaline layer substance was hydrolyzed with N/10 acetic acid at 95° C. for 3 hours. After hydrolysis the opalescence of the solution disappeared and a fine flocculent precipitate was formed. The insoluble precipitate was removed and washed with cold water, and the solution together with the washings were concentrated *in vacuo*. This concentrated solution was subsequently dialyzed against sea water. Physiological studies was performed in such concentrated solutions. The solid content of these solutions was determined by drying *in vacuo*, after they had been dialyzed against distilled water until they were free from sea water salts. In one experiment, the yield was 2.83 mg., representing 8.4 per cent of the hyaline layer substance.

It was also found that the hyaline layer substance contains a small amount of lipid. The lipid was isolated by boiling the hyaline layer substance in 95 per cent aqueous methanol for 30 minutes and subsequently extracting continuously with ether in a modified Soxlet apparatus (Abrahamson 1949) for 6 hours, the whole process being repeated three times. The ether was evaporated and the lipid weighed directly. The yield was 1.83 mg. and represented approximately 5.5 per cent of the hyaline layer substance. A qualitative test for phosphate in the lipid was made according to the method of Fiske and Subbarow (1925) and positive results were obtained. It may be said that the lipid contains phospholipid. The lipid was present in this hyaline layer preparation in such small amounts that it appears doubtful if it really forms part of the hyaline layer sub-

stance. Further studies are required to establish the finer details of this point.

Photographs were made showing the hyaline layer substance in the perivitelline space. Normally, the hyaline layer is approximately 2 microns thick and is present on the surface of the egg proper. When the eggs are washed with a molar urea solution, the hyaline layer disappears. This disappearance of the hyaline layer seems to be due to its swelling and dissolution. When the eggs are returned to sea water, the hyaline layer reappears, occupying its original position (Fig, 1*a*). If, however, unfertilized eggs are treated with cysteine or thioglycolic acid and then inseminated in sea water the hyaline layer is not reformed after the same procedure and fine precipitates appear in the perivitelline space (Fig. 1*b*). It is quite evident that these precipitates correspond to those of the hyaline layer substance precipitated with calcium. These results suggest that the hyaline layer requires disulphide bonds and calcium in order to maintain its structure. Furthermore, it seems possible that the hyaline layer has a membrane. It is interesting to note that it resembles the fertilization membrane with respect to the effect of cysteine and thioglycolic acid reported by RUNNSTRÖM *et al.* (1943) and KRAUSS (1950).



a *b*

Fig. 1. Fertilized eggs of *Hemicentrotus pulcherrimus*. (*a*) The hyaline layer reformed. (*b*) The hyaline layer substance precipitated in the perivitelline space. For further explanation see the text.

Effect of the hyaline layer substance on fertilization

Experiments were carried out to test the effect of the hyaline layer substance on re-fertilization. The hyaline layer substance was dissolved in 10 ml. of distilled water and the solution obtained was diluted ten times with sea water. A flocculent precipitate was removed by centrifugation and the supernatant was used. Consequently the concentration was not defined. The eggs were fertilized in the usual manner and put into a molar urea solution before the fertilization membrane hardened. They were left there for 2 minutes and the fertilization membrane was removed. The denuded eggs were then exposed to the solution of hyaline layer substance. After immersion for 10 minutes, the eggs were inseminated once more. Whether or not spermatozoa penetrated into the eggs at the time of the second insemination was examined after the first cleavage. It was found that heavy polyspermy occurred as frequently as in the control. This indicated that the hyaline layer substance does not inhibit re-fertilization. As mentioned above, however, the hyaline layer substance was hard to dissolve in sea water. It seems possible that the ineffectiveness of the hyaline layer substance may be due to its low concentration.

Further experiments were made with the preparations which had been autoclaved or hydrolyzed by dilute acetic acid. These preparations were dissolved completely in sea water and the active solutions contained 0.01 to 0.1 per cent dry substance. The procedure of the experiments was the same as that carried out with the untreated hyaline layer substance. The results of the experiments are presented in Table 1. From the table it is seen that polyspermic eggs were very few when the superimposed insemination was performed in the solutions of these preparations. It is clear that autoclaved or hydrolyzed preparations of the hyaline layer substance inhibit re-fertilization. In these cases, the spermatozoa were not paralyzed in the solutions and their fertilizability was maintained for a long period as in normal sea water. Furthermore, the inhibitory action of these preparations were reversible when the eggs were washed with sea water. It may be, therefore, assumed that the inhibiting substance interferes with the reaction between eggs and spermatozoa. It was also found that the inhibitory action of

these preparations is removed by addition of small amounts of sodium periodate. Sodium periodate was added to the solution 10 minutes before the eggs were added. The final concentration of sodium periodate used was 10^{-4} M. In this solution, the inhibitory action was not found and heavy polyspermy took place.

Table 1. Effect of the hyaline layer substance on re-fertilization.
Hemicentrotus pulcherrimus

Medium	Concentration of sperm in 2nd insemination	Observations 2 hrs. after 2nd insemination		
		Undivided eggs (%)	Normally divided eggs (%)	Polyspermic eggs (%)
Sea water (control)	5×10^{-3}	0	0	100
	5×10^{-4}	4	3	93
Hyaline layer substane (autoclaved)	5×10^{-3}	0	69	31
	5×10^{-4}	0	83	17
Hyaline layer substance (autoclaved) plus 10^{-4}M $NaIO_4$	5×10^{-3}	5	7	88
	5×10^{-4}	3	13	84
Hyaline layer substance (hydrolyzed)	5×10^{-3}	3	78	19
	5×10^{-4}	0	86	14
Hyaline layer substance (hydrolyzed) plus 10^{-4}M $NaIO_4$	5×10^{-3}	5	12	83
	5×10^{-4}	7	18	75

It is of interest that the inhibitory action of the hyaline layer substance does not seem to be species specific. In several experiments, the hydrolyzed hyaline layer substance from *Hemicentrotus* inhibited re-fertilization in *Pseudocentrotus* eggs.

In order to see whether or not the hyaline layer substance has an effect on normal fertilization, the following experiments were performed. The eggs were deprived of their jelly coats and placed in the solution. Ten minutes afterwards, the eggs were fertilized in it. Observations were then made on the elevation of the fertilization membrane and cleavage. It was found that in the untreated hyaline layer substance, membrane elevation was inhibited, nevertheless, fertilization is accomplished and cleavage took place in many eggs. In some experiments, the membranes were folded and the

surfaces of the eggs appeared wrinkled. If the membranes were more elevated, their toughening hardly occurred in this solution. This is similar to the action of the jelly coat substance as reported by RUNNSTRÖM and WICKLUND (1950). Furthermore, it was shown that sodium periodate completely removed the inhibitory effect of the hyaline layer substance. If the eggs were exposed to the hyaline layer substance subsequent to the addition of 10^{-4} M sodium periodate, normal membranes were elevated.

Similar results were obtained with the preparations which had been autoclaved or hydrolyzed in dilute acetic acid. These preparations, in rather high concentration (0.1 per cent), inhibit normal fertilization to 35–50 per cent. This inhibition was found to be enhanced if the eggs had been denuded of their vitelline membrane by trypsin digestion. The unfertilized eggs were treated with 0.1 per cent solution of crude trypsin (Merck). After 20 minutes, the eggs were washed with sea water and transferred to the test solution, and then inseminated in it. The results are shown in Table 2.

Table 2. Effect of the hyaline layer substance on fertilization of the trypsin-treated egg. *Hemicentrotus pulcherrimus*

Medium	Concentration of sperm	Observations 2 hrs. after insemination	
		Fertilized eggs (%)	Average (%)
Sea water (control)	10^{-3}	94–100	96
	10^{-4}	74– 89	77
Hyaline layer substance (autoclaved)	10^{-3}	39– 47	43
	10^{-4}	14– 28	20
Hyaline layer substance (autoclaved) plus 10^{-4}M $NaIO_4$	10^{-3}	92–100	95
	10^{-4}	70– 77	74
Hyaline layer substance (hydrolyzed)	10^{-3}	7– 27	14
	10^{-4}	0– 8	6
Hyaline layer substance (hydrolyzed) plus 10^{-4}M $NaIO_4$	10^{-3}	76– 96	90
	10^{-4}	73– 87	82

It will be seen that these preparations inhibit fertilization to a considerable extent. In control experiments, 77 to 96 per cent of the eggs were fertilized and cleaved without membrane formation.

Finally, experiments were carried out with the lipid which was

obtained by extraction from the hyaline layer substance. The extracted lipid did not go into solution in sea water and, therefore, the preparation was suspended in sea water at pH 8.0. The eggs were subjected to this suspension for 10 minutes and then inseminated. This experiment was performed with unfertilized eggs and fertilized eggs deprived of their fertilization membranes and hyaline layers. The results are tabulated in Tables 3 and 4. It will be seen that inhibition takes place in both fertilization and re-fertilization. In this suspension, the motility of the spermatozoa decreases within 10 minutes. However, the spermatozoa are not attacked in the period necessary for their complete penetration into the eggs. Unpublished study indicates that this period of re-fertilization is 3 to 5 minutes after the second insemination.

Table 3. Effect of the lipid extracted from the hyaline layer substance on fertilization. *Hemicentrotus pulcherrimus*

Medium	Concentration of sperm	Observations 2 hrs. after insemination	
		Fertilized eggs (%)	Average (%)
Sea water (control)	10^{-3}	98–100	99
	10^{-4}	95–100	98
Lipid suspension	10^{-3}	3– 20	10
	10^{-4}	0– 4	2

Table 4. Effect of the lipid extracted from the hyaline layer substance on re-fertilization. *Hemicentrotus pulcherrimus*

Medium	Concentration of sperm in 2nd insemination	Observations 2 hrs. after 2nd insemination		
		Undivided eggs (%)	Normally divided eggs (%)	Polyspermic eggs (%)
Sea water (control)	5×10^{-3}	0	0	100
	5×10^{-4}	0	8	92
Lipid suspension	5×10^{-3}	0	96	4
	5×10^{-4}	0	100	0

It should be noted that neither the hydrolyzate nor the lipid from the hyaline layer substance exhibits inhibiting activity after washing with sea water. In addition, the inhibition of fertilization caused by these substances is overridden by increasing the concentration of spermatozoa. These results are in contrast to the fact

that the sperm block on the egg surface is so stable that it cannot be removed by washing with sea water. An explanation may lie in the fact that the extracted substances do not attach to the egg surface as a structure, while the hyaline layer is firmly bound to the surface.

Effect of polyspermy-inducers on re-fertilization

In view of the effectiveness of the hyaline layer substance in inhibiting fertilization, it is possible that the sperm block at the fertilization may be due, in part at least, to such substances. It was of interest, therefore, to examine the relation of the sperm block on the egg surface to the mechanism which prevents polyspermic fertilization.

Previous work (NAKANO 1954) has shown that the sperm block is not formed if the eggs are activated in the presence of polyspermy-inducers. Conversely, the fully-formed sperm block is rather resistant to polyspermy-inducers. These results suggest that the formation of the sperm block on the egg surface is closely associated with the activation process. According to this concept, it might be expected that the sperm block would be formed immediately after insemination. The following experiments were planned to determine the time of formation of the sperm block on the egg surface.

Eggs were inseminated with a sperm suspension of known density and exposed to polyspermy-inducers at different times after insemination. Various reagents have been known to induce polyspermic fertilization (*cf.* CLARK 1936). Those used in the present study were acetone, ethyl urethane, nicotine and copper sulphate. These reagents were dissolved in sea water in proper concentrations and solutions were made up in every case immediately before their use. The fertilization membrane was removed in the solutions of polyspermy-inducers by repeatedly sucking and blowing the eggs with a narrow pipette. It was found that the fertilization membranes of eggs treated with these reagents are rather soft and can easily be removed. After immersion for 2 minutes, the denuded eggs were put back into sea water and washed several times, and then fresh sperm suspensions were added. When the eggs were treated within 40 seconds after insemination, inhibition of the mem-

brane elevation was often observed. In these cases, superimposed insemination was performed without removal of the membrane, as in the experiments described below. The count of polyspermic eggs was made after the first cleavage.

Table 5. Re-fertilization of once-fertilized eggs which have been treated with polyspermy-inducers at various periods after insemination. The fertilization membranes were removed mechanically after the first insemination. *Hemicentrotus pulcherrimus*

Medium	Concentration of sperm in 2nd insemination	Polyspermic eggs (re-fertilized) 30 sec. (%)	60 sec. (%)	90 sec. (%)	120 sec. (%)
Sea water (control)	5×10^{-3}	10	15	9	3
	5×10^{-4}	3	4	0	2
	not inseminated again	0	1	0	0
15% Acetone	5×10^{-3}	96	84	21	2
	5×10^{-4}	95	46	13	8
	not inseminated again	10	4	2	3
0.5% Nicotine	5×10^{-3}	100	90	42	22
	5×10^{-4}	96	84	19	17
	not inseminated again	91	4	1	9
0.5 M Urethane	5×10^{-3}	96	78	40	18
	5×10^{-4}	88	59	17	11
	not inseminated again	33	1	7	1
10^{-4} M $CuSO_4$	5×10^{-3}	96	82	29	20
	5×10^{-4}	84	37	11	12
	not inseminated again	34	7	2	10

The results of the experiments are summarized in Table 5. The data show that when the eggs are treated within about 60 seconds after insemination, re-fertilization takes place in many eggs and polyspermic features appear at the first cleavage. Under these conditions, it is apparent that the sperm block has not been formed on the egg surface. When, however, the eggs are not treated until 90 to 120 seconds after insemination, re-fertilization does not occur. In this case, the sperm block has become so stable that it cannot be removed. These results seem to indicate that there is a transitional period during which the sperm block has a low enough resistance to the reagent so that it may be easily affected. In this connection, the transitional period of the sperm block is comparable to that of the fertilization membrane. KOPAC (1941) has shown that the fertilization membrane is most sensitive to chemical and mechanical

agents during the short period immediately after it has assumed its definitive structure.

From Table 5 it is seen that when the eggs were treated with polyspermy-inducers 30 seconds after insemination, they became polyspermic without superimposed insemination. In the eggs of *Hemicentrotus*, the cortical change begins within 30 seconds after insemination. It is apparent that polyspermy-inducers are effective after the beginning of the cortical change. It should be noted that the concentrations of polyspermy-inducers used in these experiments are higher than those in which polyspermic fertilization occurs. Thus, for example, 0.05 per cent solution of nicotine, which can induce polyspermic fertilization, has a slight effect on the suppression of the sperm block of the egg surface, but pronounced effects are obtained with 0.5 per cent solution. This may be due to the difference of period treated with the polyspermy-inducer. In the case of the present experiments, the time of effectiveness of the reagent is delimited to the short period immediately after fertilization.

It was also found that when the eggs were treated with polyspermy-inducers during the transitional period of the sperm block, the percentage of re-fertilized eggs apparently increases with increasing density of spermatozoa. These results suggest that the sperm block on the egg surface is not an all-or-none mechanism.

As described elsewhere (NAKANO 1954), the strong solutions of polyspermy-inducers proved to have an effect on the development of the hyaline layer. Most of the eggs which were treated with polyspermy-inducers immediately after fertilization were lacking in normal hyaline layer and the blastomeres showed a tendency to separate from each other. From such eggs the hyaline layer substance was not obtained by the method described above. These results seem to indicate that there is a close correlation between the hyaline layer and the sperm block on the egg surface.

In these experiments, superimposed insemination was carried out after removal of the fertilization membrane. SUGIYAMA (1951) has observed that re-fertilization can occur without removing the membrane if the eggs have been treated with a Ca-Mg-free medium immediately after the first insemination. Therefore, it is of interest to ascertain whether the eggs can be re-fertilized without removing

the membrane when treated with polyspermy-inducers.

The experimental procedure was the same as that described above, except that the fertilization membrane was not removed. Some of the results in which nicotine and urethane were used are shown in Table 6. These results suffice to show that re-fertilization do take place without removal of the membrane if the eggs have been treated with polyspermy-inducers immediately after the first insemination. Under these conditions, the membrane of many eggs appears to be somewhat soft, inasmuch as it may be thinner than the membrane of control eggs. Too long a stay of the eggs in sea water before their transference to polyspermy-inducers results in the failure of re-fertilization, since the membrane becomes hard enough to prevent the penetration of the spermatozoa. In this case too, it is clear that the sperm block undergoes a transitional period before attaining its complete effectiveness. This period, however, does not coincide with the transitional period of membrane toughening which continues for at least 2 minutes.

Table 6. Re-fertilization of once-fertilized eggs which have been treated with polyspermy-inducers at various periods after insemination. The fertilization membranes were not removed.
Hemicentrotus pulcherrimus

Medium	Concentration of sperm in 2nd insemination	Polyspermic eggs (re-fertilized) 30 sec. (%)	60 sec. (%)	90 sec. (%)	120 sec. (%)
Sea water (control)	5×10^{-3}	3	3	1	2
	5×10^{-4}	1	2	4	1
	not inseminated again	2	0	0	1
0.5% Nicotine	5×10^{-3}	100	72	6	1
	5×10^{-4}	100	45	5	3
	not inseminated again	95	8	4	3
0.5 M Urethane	5×10^{-3}	77	43	9	5
	5×10^{-4}	23	7	8	5
	not inseminated again	33	1	7	1

The results of these experiments are summarized graphically in Fig. 2. The curves shown in the figure represent the results obtained in two sets of experiments in which urethane was used as the polyspermy-inducer.

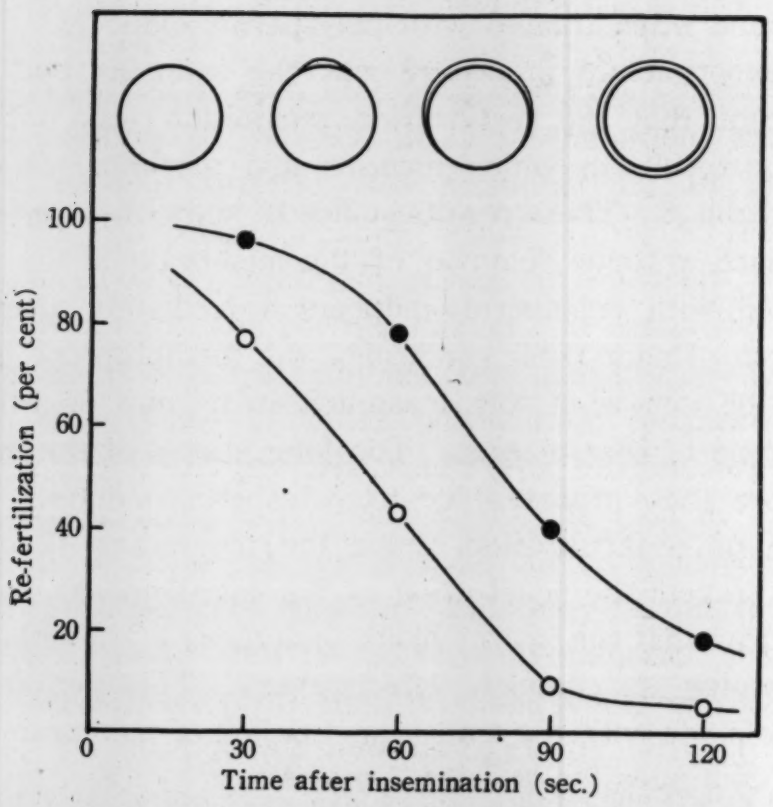


Fig. 2. Re-fertilization of once-fertilized eggs which have been treated with 0.5 M urethane at various periods after insemination. ○, with fertilization membranes; ●, without fertilization membranes. Upper diagram shows successive phases of the membrane elevation in the fertilized egg.

It is seen that the longer the time is allowed to elapse after insemination, the less re-fertilization. This means that the sperm block becomes so stable that it cannot be affected by the reagent. Under the conditions of the experiments, it may be assumed that the percentage of re-fertilized eggs represents the state of the sperm block. According to this assumption, the sperm block of the egg surface is completed in less than 120 seconds after insemination. If, as demonstrated above, the sperm block of the egg surface is associated with the hyaline layer, the latter must appear immediately after fertilization. In harmony with this, recent observations have shown that the secretion of the hyaline layer begins 40 seconds after insemination (Ishida 1951, Moore 1951).

By a similar logic, the sperm block of the fertilization membrane is completed within 60 seconds after insemination. At the end of this period, the fertilization membrane begins to separate from the surface of the egg. It is evident that the sperm block of

the fertilization membrane is formed before separation of the membrane. Even if the fertilization membrane does not separate completely, the cortical change spreads over the entire periphery of the egg. Therefore, the formation of the sperm block appears to be associated with the cortical change. These results are in agreement with those reported by ROTHSCHILD and SWANN (1952) in over-insemination experiments.

Effect of enzymes on re-fertilization

Experiments were performed to determine the effect of some enzymes on re-fertilization. Eggs were fertilized and the fertilization membrane was mechanically removed, and the denuded eggs were treated with 0.5 per cent solution of enzymes for 10 minutes. After this they were repeatedly washed with sea water and fresh sperm suspensions were added. In each case, the re-fertilizability of treated eggs was compared with that of otherwise identical untreated ones.

Table 7. Re-fertilization of once-fertilized eggs which have been treated with proteolytic enzymes after removal of the fertilization membrane.
Hemicentrotus pulcherrimus

Enzymes	Concentration of sperm in 2nd insemination	Observations 2 hrs. after 2nd insemination		
		Undivided eggs (%)	Normally divided eggs (%)	Polyspermic eggs (%)
Sea water (control)	5×10^{-3}	1	95	4
	5×10^{-4}	5	90	5
	not inseminated again	0	97	3
0.5% Trypsin	5×10^{-3}	0	3	97
	5×10^{-4}	4	20	76
	not inseminated again	0	97	3
Acid sea water (pH 4.5)	5×10^{-3}	3	86	11
	5×10^{-4}	0	92	8
	not inseminated again	1	93	6
0.5% Pepsin in acid sea water (pH 4.5)	5×10^{-3}	0	6	94
	5×10^{-4}	0	26	74
	not inseminated again	0	93	7

The results of the experiments performed with proteolytic enzymes are shown in Table 7. The pH of the trypsin solution was 8.0 and that of the pepsin solution was 4.5. It is seen that re-fertilization can be induced by treating eggs with such enzymes. Of particular interest is the fact that these enzymes inhibit the forma-

tion of a typical hyaline layer and the blastomeres more or less fall apart. This affords indirect evidence for the association of the sperm block with the hyaline layer.

In these experiments, however, the enzymes used were crude preparations (Merck). It should be noted that crystalline trypsin (Worthington Biochemical Sales Co.) has no apparent effect, either on the sperm block or on the hyaline layer, at the concentrations (0.1 to 0.01 per cent) used in these experiments. The dissolution of the hyaline layer brought about by the crude trypsin is therefore probably due to a component other than trypsin.

The fact that the hyaline layer substance is a lipid-carbohydrate-protein complex suggests that the hyaline layer might be attacked by mucopolysaccharases. To test this, bull testis hyaluronidase (Motida Co.), lysozyme (Nutritional Biochemicals Corp.) and an extract from liver of *Charonia* were used. These enzymes were found to be ineffective in dissolving the hyaline layer, as well as in making fertilized eggs re-fertilizable.

Discussion

The association between the hyaline layer and the sperm block on the surface of the fertilized egg raises several interesting questions in connection with the mechanism of blocking fertilization and with problems of fertilization generally. Those which will be discussed here are: (1) the nature of the hyaline layer, (2) the time relationship of the block formation and (3) the possible mechanism which prevents polyspermy.

1. It was first shown by Moore (1930) that the hyaline layer has the properties of a calcium proteinate, and later its protein nature was established by Runnström *et al.* (1943), who showed that it is digested by proteolytic enzymes, such as trypsin. On the other hand, Bohus Jensen (1950, 1953) demonstrated that pretreatment with crystalline trypsin does not interfere with the formation of the hyaline layer. This author suggests that destruction of the hyaline layer by crude trypsin may be due to a component other than trypsin. Recently, Moore (1952) reported that crystalline trypsin causes dissolution of the hyaline layer, resulting in a loose

connection of the blastomeres. He states, however, that the blastomeres are held together by the intercellular structure. It would appear from this that crystalline trypsin does not completely dissolve the hyaline layer. In the present experiments, it was confirmed that crude trypsin is more effective in dissolving the hyaline layer.

MONNÉ and SLAUTTERBACK (1950) showed that the hyaline layer could be stained with fuchsin-sulphite when the combined phenylhydrazine fuchsin-sulphite method is applied. As they also described similar staining properties of the jelly coat, it was inferred that the hyaline layer probably contains acid polysaccharides. The present experiments provide further evidence that the hyaline layer contains mucopolysaccharides. In addition, the hyaline layer substance is associated with lipid. The presence of carbohydrate and lipid in the materials of the cell surface has been shown in those of red corpuscles (MOSKOWITZ and CALVIN 1952) and yeast cells (NORTHCOTE and HORNE 1952).

On the other hand, it has already been stated that the hyaline layer has a protein nature. In this connection, the results of experiments with sulfhydryl compounds are significant. These show that the hyaline layer may require disulphide bonds in order to maintain integrity. They also suggest that the hyaline layer has a membrane-like structure. It seems that proteolytic enzymes may have an effect, in part, on the membrane of the layer. RUNNSTRÖM *et al.* (1943) have also reported that thioglycolic acid inhibits the formation of a typical hyaline layer.

Although the hyaline layer substance is resistant to solution in sea water, autoclaved or hydrolyzed preparations are dissolved to a considerable extent. It was found that these preparations have the power of inhibiting both fertilization and re-fertilization. Inasmuch as the action of the substance is easily removed by the addition of sodium periodate in low concentrations, it resembles heparin or fucoidin, as reported by RUNNSTRÖM and KRISZAT (1950) and HARDING (1951). It seems likely that the action of the hyaline layer substance, at least in part, depends on a polysaccharide component. Recently WICKLUND (1954) studied the influence of a number of natural and synthetic substances on fertilization and membrane for-

mation. She divided the inhibitory substances into two groups: (1) those inactivated by treatment with periodate, and (2) those which resist periodate. The periodate inactivation is due to the polysaccharide nature of the inhibitors of group 1. The same author suggested that the inhibitor belonging to group 1, particularly the fertilization inhibitor from *Fucus* (FeInh (Fu)), is analogous to a natural inhibitor present in the mature, unfertilized egg. According to the concept developed by RUNNSTRÖM (1949, 1952), a polysaccharide serves as a natural inhibitor of enzymes and mediates the block against activation in the unfertilized egg. Upon fertilization the activity of this inhibitor would have to be removed. It seems probable that the hyaline layer substance is closely related to the natural inhibitor present in the egg.

In addition to these results, a correlation has been shown between the sperm block on the surface of the fertilized egg and the presence of the hyaline layer. As noted earlier in this discussion, the hyaline layer is attacked to some extent by proteolytic enzymes. Under these conditions, re-fertilization can take place.

Conversely, if the eggs were exposed to polyspermy-inducers after fertilization, these reagents seem to interfere with the development of a normal hyaline layer, when re-fertilization can be induced with relatively low concentrations of sperm. From these results it may be concluded that the sperm block on the egg surface is associated with the hyaline layer. Recently, HAGSTRÖM and his collaborators (1954 a, 1955 a, b) have reached the same conclusion in their experiments with trypsin-treated eggs and with partially fertilized eggs.

2. The time relationship of the formation of the sperm block at fertilization of the sea urchin egg has been worked out in detail by ROTHSCHILD and SWANN (1949–1953), who proposed that at fertilization there is a fast but incomplete block with a conduction time of less than 2 seconds and a slower block which finally makes the egg impermeable to spermatozoa. It was also suggested that the visible cortical change is a reflection of the slow component of the sperm block.

The present experiments support the assumption as to the so-called slow block. These show that the sperm block in the ferti-

lized egg becomes resistant to polyspermy-inducers after 60 seconds, by which time the cortical change has completely covered the egg surface. They further indicate that the sperm block of the denuded egg is also achieved within about 90 seconds after insemination. In agreement with this, the growth of the hyaline layer can be observed immediately after fertilization. Thus it appears that both sperm blocks reported here corresponds to the slow and complete block of ROTHSCHILD.

It was also suggested that there is a transitional period, that is, an intermediate period between the initial block and the complete block. During this period, polyspermy-inducers can interfere with the formation of the complete block. The presence of such an inhibitor-sensitive period was shown by TYLER and SCHULTZ (1932) in *Urechis* eggs. They found that the reversal of fertilization is possible, if the eggs are exposed to acid sea water within 3 minutes after fertilization. In this case, however, the sperm block is not affected by the treatment.

3. It is most probable that in the fertilized egg there are two sperm blocking mechanisms. One of them is, of course, the fertilization membrane and the other is the hyaline layer covering the surface of the egg. The present experiments have shown that these sperm blocks are affected by polyspermy-inducers, if the eggs are treated immediately after fertilization. This suggests that there is a close correlation between the sperm block in the fertilized egg and the sperm block which prevents polyspermic fertilization.

The possibility that the sperm block might be attacked by nicotine was discussed by ROTHSCHILD and SWANN (1950, 1953). These authors did not find any delay in the conduction time of the cortical color change under the action of nicotine and concluded that nicotine mainly abolishes the fast, incomplete block. If, however, the cortical color change is unaffected by nicotine, it must be concluded that nicotine inhibits reactions occurring after this change. A support to this idea is the fact that the toughening of the fertilization membrane is markedly delayed when the eggs have been treated with strong solutions of polyspermy-inducers. In some cases, the formation of the hyaline layer is inhibited to a certain extent. Such eggs are receptive to re-insemination as judged by their cleavage

pattern. Of possible significance in connection with these results is the report by RUNNSTRÖM and WICKLUND (1949), that the jelly coat substance prevents the merging of the cortical material with the vitelline membrane. This was deduced as the result of observations that the fertilization membrane does not toughen in the presence of the jelly coat substance. Under this condition, there is a definite tendency toward increased polyspermy (RUNNSTRÖM and KRISZAT 1950).

The evidence so far available suggests that the processes connected with the toughening of the fertilization membrane play a role in the prevention of polyspermy. The role of the fertilization membrane was first noticed by FOL (1879) who believed that its progressive elevation was the block to polyspermy. Although the appearance of the fertilization membrane takes place about one minute, the changes preparatory to its elevation must begin at an earlier stage. The cortical change, which is the first sign of fertilization, requires about 20 seconds to pass over the egg surface after adhesion of the fertilizing spermatozoon. The change of the membrane may occur during this phase. It may be suggested that merging of the membrane with the cortical material constitutes the block to polyspermy. Recently, ROTHSCHILD (1953) has proposed that the slow block is attributable to the fertilization membrane. These assumptions are supported by the fact that if the vitelline membrane of the unfertilized egg has been digested by trypsin, polyspermic fertilization can take place (BOHUS JENSEN 1953 and HAGSTRÖM and HAGSTRÖM 1954 b). The same results have been obtained by the present author.

The nature of the fast incomplete block is as yet quite unknown. RUNNSTRÖM *et al.* (1944, 1945, 1946) have shown that the sperm lysin extracted from spermatozoa or eggs has the capacity of blocking fertilization. The sperm lysin is apparently a highly non-specific, heat-stable substance of lipid nature and low molecular weight. It has been suggested by ISHIDA (1950) that lipolytic enzymes may play a role in preventing polyspermic fertilization. He considered that fatty acids split by such enzymes may constitute the fast block to polyspermy. If, as has been demonstrated in the present study, lipid is associated with the hyaline layer, it seems

possible that lipid may be connected with the natural mechanism which prevents polyspermy.

Recently, ALLEN and HAGSTRÖM (1955 a, b) have thrown doubt on the existence of the fast block to polyspermy. They have shown that the unfertilized surface of the partially fertilized egg can be fertilized at light sperm densities. This suggests that no irreversible fast block propagates before passage of the visible cortical change. However, a possibility must be considered that the fast incomplete block has a sufficiently low resistance to agents so that it may be easily removed. The investigation of this possibility must be the subject of future studies.

SUMMARY

1. Fertilized eggs of the sea urchin can be fertilized a second time if they are washed in a molar urea solution after removal of the fertilization membrane. This suggests that the sperm block is removed from the egg surface in the urea solution. The presence of the substance constituting the block is demonstrated in such a solution and the substance is designated "hyaline layer substance". The hyaline layer substance is non-dialyzable and precipitable by ammonium sulphate. It gives positive protein reactions and contains sugar and possibly lipid. These properties indicate that the hyaline layer substance is a lipid-carbohydrate-protein complex.

2. The hyaline layer substance does not inhibit fertilization, probably because of its low solubility in sea water. However, if the substance is heated or hydrolyzed in dilute acid solution, it becomes soluble in sea water and shows activity in preventing fertilization. This inhibitory action depends on a polysaccharide component, because the addition of periodate in low concentration quickly removes the activity of the preparation. The extracted lipid is also proved to have the power of inhibiting both fertilization and re-fertilization.

3. Polyspermy-inducers are shown to have an effect on the sperm block of the fertilized egg. Fertilized eggs deprived of their membranes can be re-fertilized if they are subjected to polyspermy-inducers within 60 seconds after insemination. Such eggs lack a

normal hyaline layer. When the eggs are not treated with polyspermy-inducers until 90 to 120 seconds after the first insemination, the sperm block has become so stable that it cannot be removed by such treatment. It is suggested that there is a transitional period which can be artificially reversed or suppressed. During this period, the sperm block has a sufficiently low resistance to polyspermy-inducers so that it may be easily rendered ineffectual. Other experiments, in which the eggs have been exposed to proteolytic enzymes after fertilization, show similarly that both the sperm block and the hyaline layer are attacked by the enzymes. It may be concluded that the sperm block on the egg surface is associated with the hyaline layer. In the case of eggs with the fertilization membrane, the sperm block is also sensitive to polyspermy-inducers within a short period after insemination. Since polyspermy-inducers have an inhibitory effect on the toughening of the fertilization membrane, it is suggested that changes of the fertilization membrane also play a role in the prevention of polyspermy.

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Addendum

After this manuscript was submitted, two papers appeared concerning re-fertilization and polyspermy, one by Tyler, Monroy and Metz[1] and another by Hagström and Allen[2]. Tyler *et al.* have shown that re-fertilization takes place without treatment of Ca-Mg-free media in eggs of *Lytechinus pictus* and *L. variegatus*. However, as they have pointed out, this difference may be due to differences in the species of echinoid used. Hagström and Allen have described the detailed mechanism of nicotine-induced polyspermy and they have pushed the idea that the hyaline layer plays a role in the establishment of the block to polyspermy. While their results agree well in many respects with the present data, there are some points of difference. It seems likely that changes in the fertilization membrane are rather important for the prevention of polyspermy.

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1) Tyler, A., A. Monroy and C. B. Metz, 1956. Fertilization of fertilized sea urchin eggs. Biol. Bull., *110*, 184-195.

2) Hagström, B. E. and R. D. Allen, 1956. The mechanism of nicotine-induced polyspermy. Exp. Cell Res., *10*, 14-24.

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THE CHANGES IN PHOSPHORUS COMPOUNDS DURING EMBRYONIC DEVELOPMENT OF THE SILK WORM, *BOMBYX MORI*

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INTRODUCTION

The transformation of various phosphorus (P) compounds during metamorphosis and embryonic development of insects has recently been studied by several workers. LEVENBOOK (1953) has reported the changes in P compounds during metamorphosis of a blow fly, *Calliphora erythrocephala*. LU and BODINE (1953) have analysed the distribution of P in the developing egg of the grasshopper, *Melanoplus differentialis*, and have shown that there occurs striking changes in both the distribution and the amount of P compounds, *i.e.*, a marked increase of nucleic acid P and a corresponding decrease of lipid P and acid soluble P. However, the mechanism controlling these phenomena are as yet not well understood.

The present study has been undertaken with a hope to contribute additional information to P transformation in the developing egg. The eggs of silk worm, *Bombyx mori*, seem to be one of the most convenient materials for such studies for the following reasons. First the development proceeds very uniformly, so that one can easily obtain the eggs at a desired developmental stage. Second, since the egg is a cleidoic or a closed type system, the total amount of P present in the egg is constant throughout development. Third, in the silk worm of a bivoltine race, two types of egg, the diapausing and non-diapausing, can be produced at will, for the type of eggs is determined by the temperature and light condition given to the egg stage. Furthermore, the diapause egg can be artificially converted to non-diapause type by immersing the egg in warm dilute HCl for

several minutes. It is probable that comparison of the phosphorus metabolism among these three kinds of eggs may afford an insight not only into the mode of transformation of P compounds, but also into the mechanism of the metabolic adjustment in the diapause egg.

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MATERIALS AND METHODS

The eggs of Chinese bivoltine race were used as materials. Non-diapause eggs were obtained from the moths which were incubated during the egg stage at 15° C. in the dark. Diapause eggs were laid by the moths which had developed from the eggs at 25° C. under illumination. In order to reactivate the diapause eggs, they were immersed into hot dilute HCl (specific gravity 1.075, temperature 46° C.) for 5 minutes at 20th hour after oviposition, after which they were rinsed carefully with running water and dried quickly. Hereafter these eggs will be referred to as "artificial non-diapause egg." Unless otherwise stated, the eggs were incubated at 25° C. throughout development.

Exactly 360 eggs of a known age, the total weight of which was 200±0.5 mg. at the time of oviposition of diapause egg, were employed for each determination.

At the beginning, the P compounds in the eggs were divided into the following four fractions: trichloracetic acid (TCA) soluble P, lipid P, total nucleic acid P and phosphoprotein P.

Basing upon the techniques of BARTH and JAEGER (1947) and of SCHNEIDER (1945), the extraction and separation of these diffrent P fractions were carried out according to the procedure shown in the scheme (p. 169). A further analysis of acid soluble P was carried out by the method of LEPAGE (1949) with some modifications. Determination of P were made by the method of LOHMANN and JENDRASSIK (1926). "Apparent inorganic P" (P_0) includes orthophosphate liberated from very labile P compound, such as phosphagen, by the procedure employed. "True inorganic P" (inorganic P) was measured after its precipitation with $CaCl_2$-$Ca(OH)_2$. P_{10} and P_{180} represent the apparent inorganic P values after hydrolysis by N HCl for 10 and 180

Scheme of extraction and fraction

360 eggs were homogenized in 1 ml. 75 per cent trichloracetic acid (TCA), added 7 ml. cold distilled water, kept for 15 minutes, centrifuged. (0–5° C. throughout)

- Residue: reextracted with 2 ml. cold 7.5 per cent TCA, centrifuged (0–5° C.)
- Supernatant$_1$
- Residue: suspended in a mixture of 8 ml. cold distilled water and 3 ml. 95 per cent ethanol, centrifuged (repeated twice)
- Supernatant$_2$: combined to sup.$_1$, used to fractionate acid soluble P. (see the text for details)
- Residue: extracted with 5 ml. 95 per cent ethanol, centrifuged
- Supernatant: discarded
- Residue: extracted with 5 ml. ethanol-ether (3 : 1) boiled at 85° C. for 3 minutes, cooled, centrifuged (repeated twice)
- Supernatant$_{L1}$
- Residue: suspended in 1.2 ml. cold distilled water and 1.3 ml. cold 5 per cent TCA, centrifuged
- Supernatant$_{L2 \text{ and } L3}$: combined to sup.$_{L1}$, used to determine lipid P
- Residue: suspended in 5 ml. 5 per cent TCA, boiled at 90° C. for 15 minutes, cooled, centrifuged
- Supernatant: discarded
- Residue: suspended in 2.5 ml. 5 per cent TCA, centrifuged
- Supernatant$_{N1}$
- Residue: dissolved in 5 ml. 2 per cent NaOH, heated at 100° C. for 10 minutes, cooled, centrifuged
- Supernatant$_{N2}$: combined to sup.$_{N1}$, used to determine nucleic acid P
- Residue: discarded (no phosphorus present in this residue)
- Supernatant: used to determine protein P

minutes respectively. Thus $P_{10}-P_0$ and $P_{180}-P_{10}$ respectively give the amount of easily and difficultly hydrolyzable P esters. In several instances, the nucleic acid P was further divided into ribonucleic acid P (RNAP) and desoxyribonucleic acid P (DNAP). For the measurement of DNAP, the method of DISCHE's color reaction (1930) was employed, assuming that the ratio of purine and pyrimidine base in DNA of the silk worm egg is approximately 1 throughout development. The amount of RNAP was estimated by difference between total nucleic acid P (TNAP) and DNAP. Further details of the methods will be described later in connection with individual experiments.

RESULTS

Transformation of P Compounds in the Developing and Diapause Egg

Non-diapause Egg. The total period for embryonic develpoment was about 230 hours at 25° C.

The variation in acid soluble P, lipid P, total nucleic acid P and protein P with respect to the development is shown in Figure 1. Acid soluble P decreased steadily from 1.49 μg. per egg at the time of oviposition to 1.06 μg. per egg at the time of hatching.

Lipid P and total nucleic acid P, on the other hand, increased with development from 0.16 to 0.32 μg. and from 0.08 to 0.38 μg. per egg respectively; thus the sum of increase in these two fractions approximately coincides with the decrease in acid soluble P.

Protein P represents a very small fraction of total P, *i.e.*, 0.07 μg. per egg, which shows no marked change throughout the development.

The changes in various fractions of acid soluble P are shown in Figure 2. The inorganic P increased progressively up to he time of blastokinesis which occurred at about 4 days of development. From this point it decreased rapidly. The curves for the easily and difficultly hydrolyzable P ($P_{10}-P_0$ and $P_{180}-P_{10}$) were the highest at the time of oviposition and decreased to minimum values about half way through the development. During the later period, the former fraction increased gradually, while the latter changed little. The non-hydrolyzable P ($P_{\text{acid soluble}}-P_{180}$) decreased steadily with development,

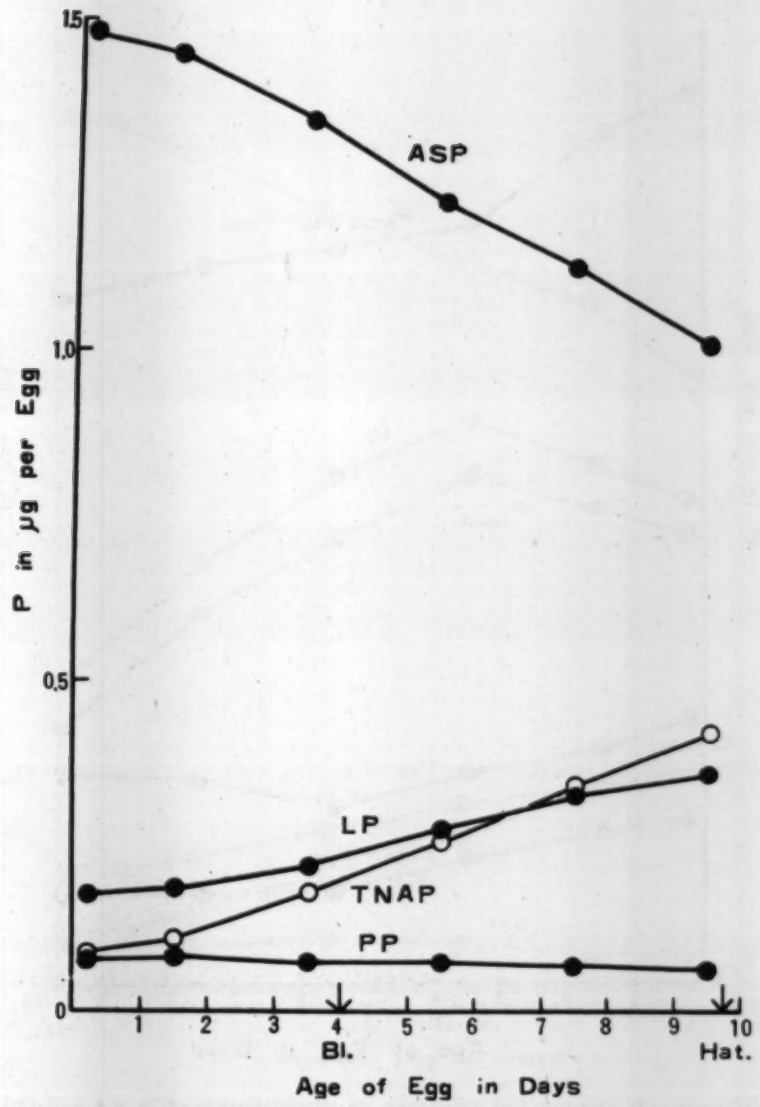


Fig. 1. Changes in acid soluble P (ASP), total nucleic acid P (TNAP), lipid P (LP) and protein P (PP) of the non-diapause egg of silk worm during development.
Bl.: blastokinesis, Hat.: hatching.

the decrease being more pronounced before blastokinesis than after it. Thus, the decrease of over all acid soluble P, shown in Figure 1, can be attributed to the decrease of easily and difficultly hydrolyzable and non-hydrolyzable P in the early developmental period, but to that of inorganic P and non-hydorlyzable P in the later period.

Before going further, it will be worth noting that the difference between inorganic P and P_0, which is considered to indicate the amount of phosphagen, scarcely fluctuates throughout the development.

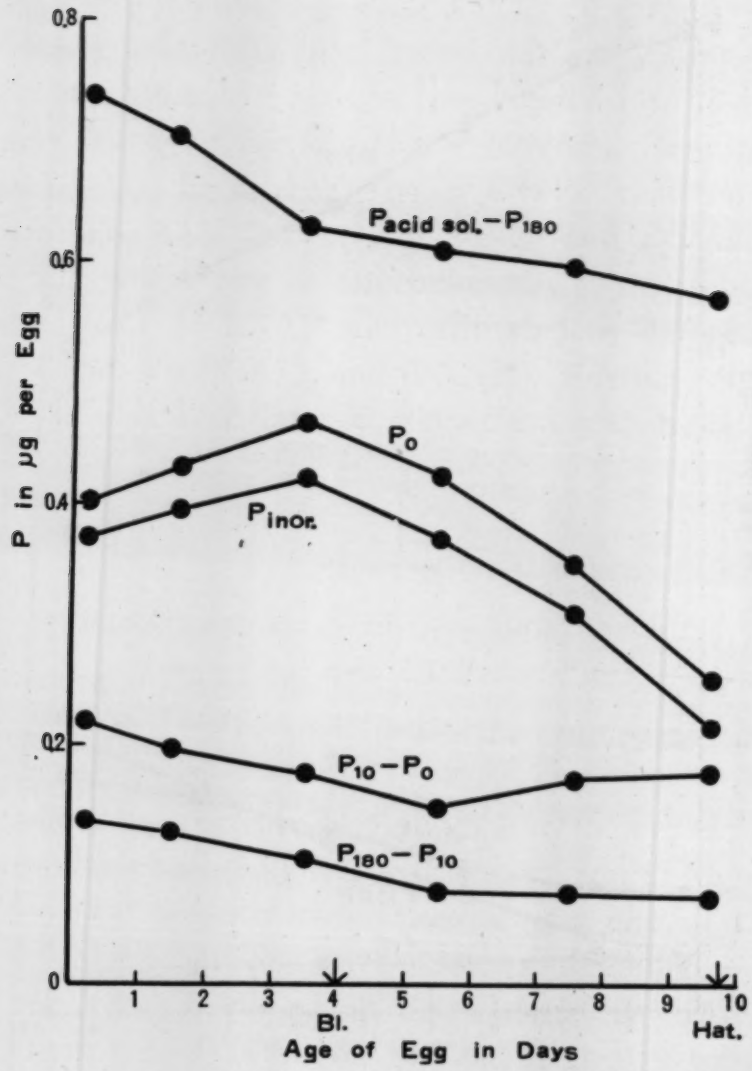


Fig. 2. Change in various components of acid soluble P of the non-diapause egg of silk worm during development.
Bl.: blastokinesis, Hat.: hatching. P_0 and $P_{inor.}$ represent "apparent" and "true" inorganic P respectively. $P_{10}-P_0$, $P_{180}-P_{10}$ and $P_{acid\ sol.}-P_{180}$ represent respectively easily, difficultly and non-hydrolyzable P.

Artificial non-diapause egg. The eggs, destined for diapause, were treated with warm dilute HCl at 20 hours after oviposition. All the eggs, thus treated, developed without diapause and hatched at about 230 hours after the acid treatment.

As shown in Figure 3, the variation in acid soluble P, lipid P, total nucleic acid P and protein P was essentialy similar to that found in the non-diapause egg, except that the amount of acid soluble P at the onset of the development was slightly lower in the artificial

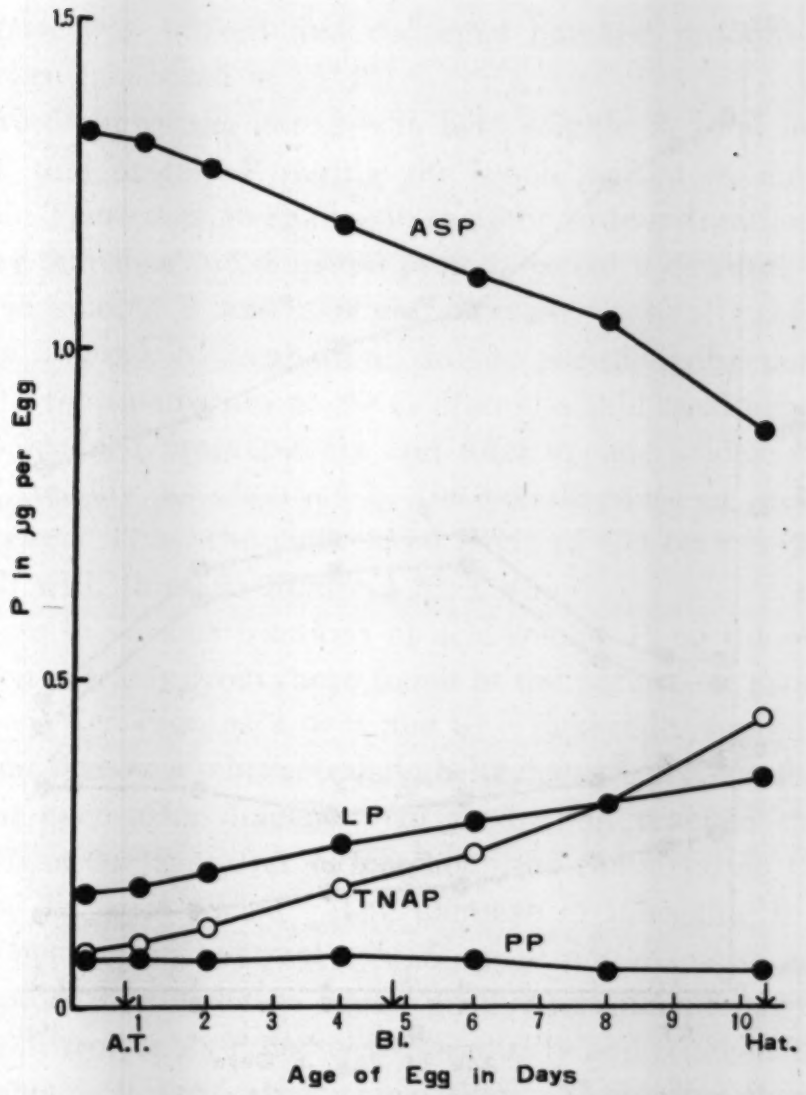


Fig. 3. Changes in acid soluble P, total nucleic acid P, lipid P and protein P of the artificial non-diapause egg of silk worm during development.
A.T.: acid treatment, Bl.: blastokinesis, Hat.: hatching.

non-diapause eggs than in the non-diapause eggs.

The changes in various fractions of acid soluble P are indicated in Figure 4. By comparing Figures 2 and 4, it is evident that there is no essential difference between the natural non-diapause and artificial non-diapause eggs. However, a close examination reveals the following points; at the time of oviposition the amount of inorganic P is about 30 per cent higher in the former than in the latter. In artificial non-diapause egg, inorganic P increases progressively up to the time of blastokinesis, but thereafter it remains nearly constant

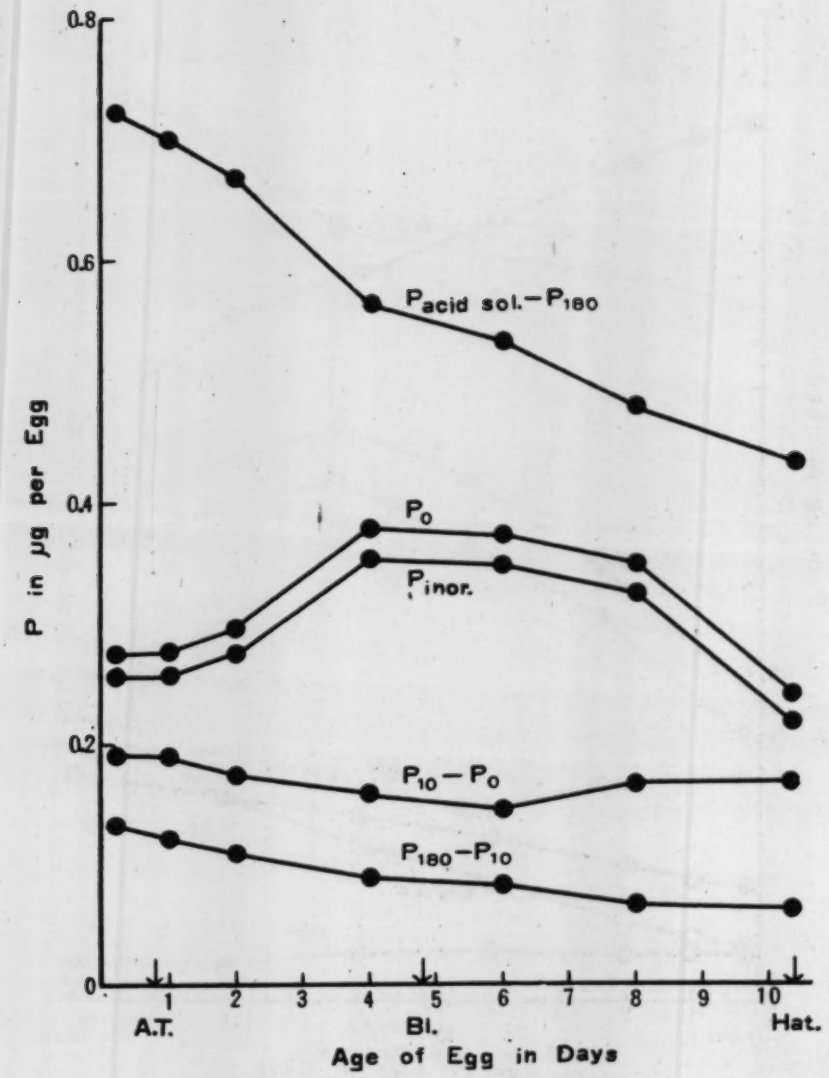


Fig. 4. Change in various components of acid soluble P of the artificial non-diapause egg of the silk worm during development.
A.T.: acid treatment, Bl.: blastokinesis, Hat.: hatching.

during about two days (in stead of decreasing at once, as was found in non-diapause eggs). Non-hydrolyzable P decreases from 0.7 to 0.4 μg. per egg through development, the decrease being about twice as great as that of natural non-diapause egg.

Diapause egg. The eggs were kept at 25° C. for 30 days after oviposition. During this period, they entered diapause and morphogenetic development was completely arrested. Thereafter they were kept at room temperature for 165 days (from early summer to winter) and then in a refrigerator at 5° C. for 65 days. By the stimulus of

cold, diapuse was broken and the eggs hatched promptly and uniformly when incubated at 25° C.*

Figure 5 shows the changes in acid soluble P, total nucleic acid P, lipid P and protein P during the whole period of diapause and activation. There was no change in the P of all four fractions throughout the pre-diapause and diapause period, except a detectable decrease in the acid soluble P and a slight increase in total nucleic acid P during the first day of incubation. In the post-diapause period which began with the incubation at 25° C. after the cold treatment, the acid soluble P declined progressively and total nucleic acid P and lipid P increased steadily, as observed in the non-diapause or artificial non-diapause egg. Thus, the right hand parts of the curves of Figure 5 agree well with those in Figure 1 or 3.

Changes in various fractions of acid soluble P, on the other hand, differed considerably from those found in the natural or artificial non-diapause egg (*cf.* Figures 2 or 4 and 6). Especially inorganic P and phosphagen P showed characteristic behavior. Inorganic P continued to increase even after diapause had set in and reached the highest value at about 30 days after oviposition, and remained so throughout the rest of diapause period. The increase of inorganic P amounted to 0.3 μg. per egg in contrast to 0.05 μg. of non-diapause eggs or 0.1 μg. of artificial non-diapause eggs. Easily and difficultly hydrolyzable P and non-hydrolyzable P decreased gradually and reached the lowest value at about 50 days after oviposition. Thereafter they changed little until the termination of diapause. When development was resumed by the cold treatment and subsequent incubation at 25° C., non-hydrolyzable P decreased steadily with the development as was observed in the natural or artificial non-diapause eggs, while inorganic P showed no marked change during the first 3 days of incubation and then declined rapidly till the time of hatching. Easily and difficultly hydrolyzable P increased gradually and then showed fluctuation similar to those found in the non-diapause eggs.

Now let us consider the behavior of phosphagen of the diapause egg. As was described previously, the difference between the inorganic P and P_0 is considered to represent the amount of phosphagen

* In order to avoid abrupt change of temperature, the eggs, taken out from the refrigerator, were kept at 15° C. for one day and then transferred to 25° C. They hatched 245 hours after the commencement of incubation.

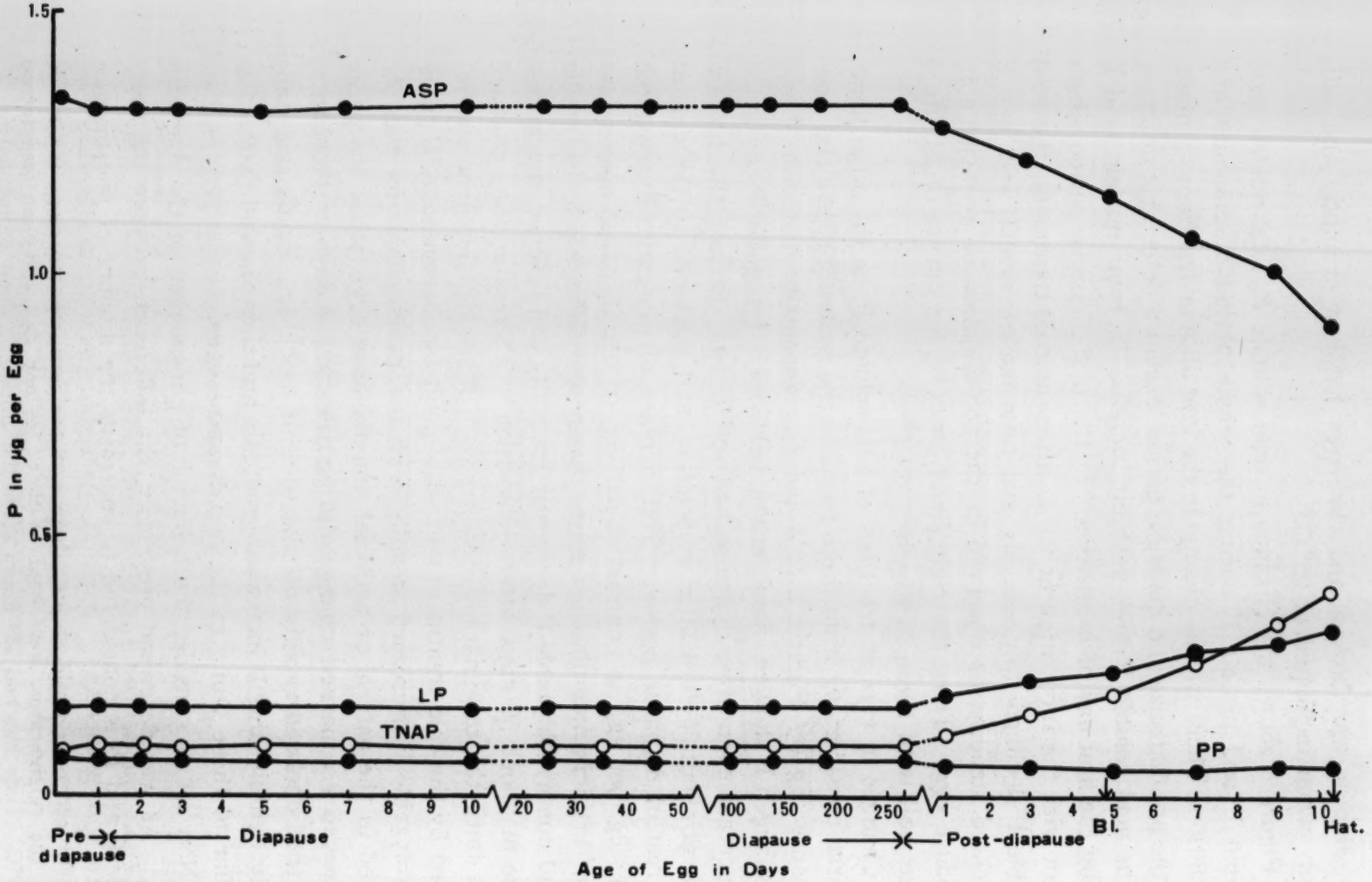


Fig. 5. Changes in acid soluble P, total nucleic acid P, lipid P and protein P of the egg of silk worm during diapause and activation. The initiation of diapause is tentatively taken as the time when oxygen uptake of the egg begins to fall (see the text). Bl.: blastokinesis, Hat.: hatching.

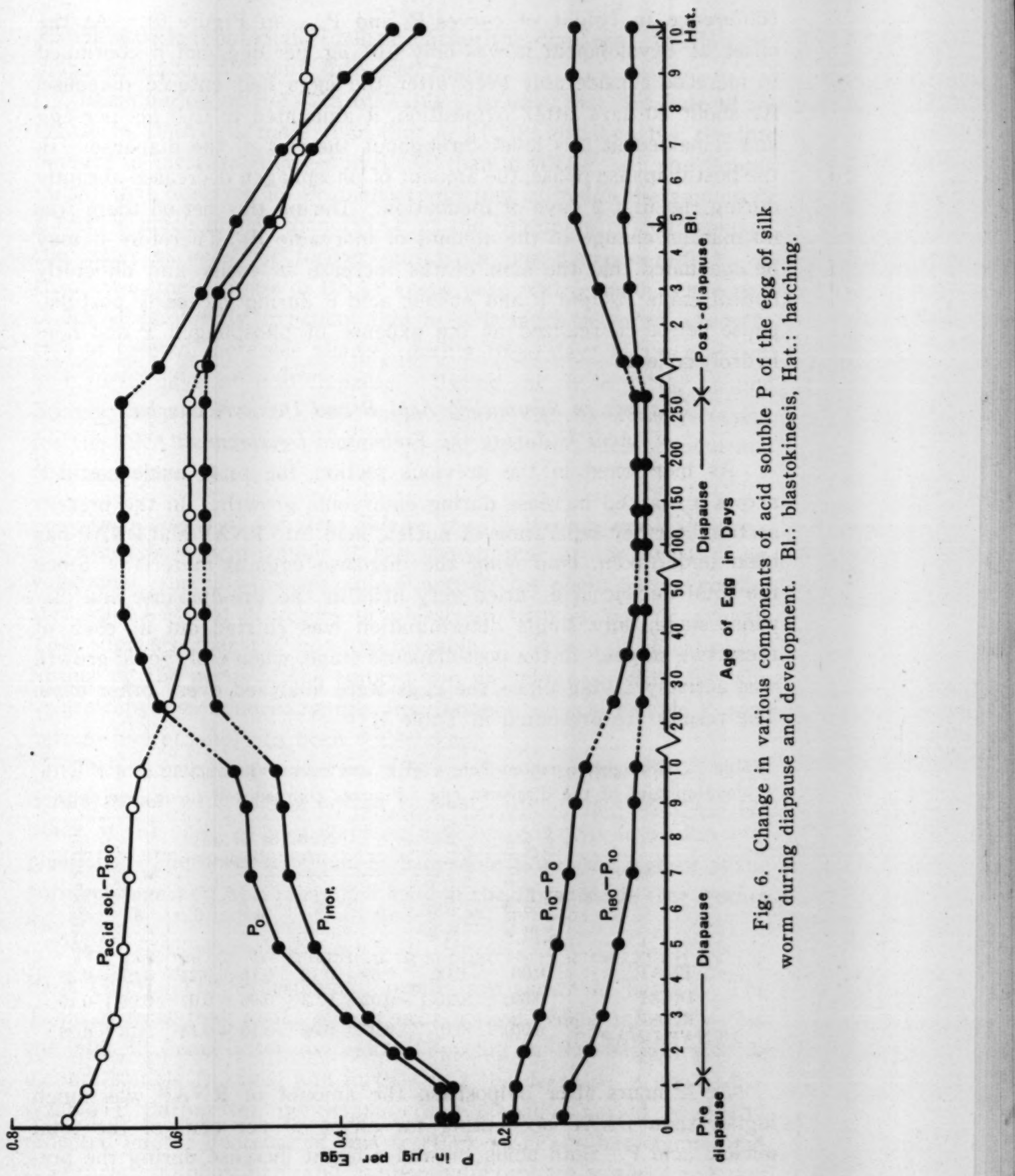


Fig. 6. Change in various components of acid soluble P of the egg of silk worm during diapause and development. Bl.: blastokinesis, Hat.: hatching.

(difference in height of curves P_0 and $P_{1nor.}$ in Figure 6). At the onset of development it was only 0.01 μg. per egg, but it continued to increase considerably even after the eggs had entered diapause. At about 50 days after oviposition, it amounted to 0.09 μg. per egg and remained at this level throughout the rest of the diapause. In the post-diapause phase, the amount of phosphagen decreased abruptly during the first 3 days of incubation. During this period there was no marked change in the amount of inorganic P. Therefore it may be concluded that the sum of the increase in easily and difficultly hydrolyzable P, lipid P and nucleic acid P during the early post-diapause period is realized at the expense of phosphagen P and non-hydrolyzable P.

Changes in Ribonucleic Acid P and Desoxyribonucleic Acid P during the Embryonic Development

As mentioned in the previous section, the total nucleic acid P shows a marked increase during embryonic growth. In the present section; further separation of nucleic acid into RNAP and DNAP has been undertaken, employing the diapause eggs as material. Since the total nucleic acid varied very little in the pre-diapause and diapause stage, only single determination was carried out at each of these two stages. In the post-diapause stage, when embryonic growth was actively taking place, the eggs were analyzed every other days. The results are presented in Table 1.

Table 1. Changes in ribonucleic acid P and desoxyribonucleic acid P with development of the diapause egg. Figures represent P in μg. per egg.

	Pre-diapause 12 hrs. after oviposition	Diapause 125 days after oviposition	Number of days after diapause					Hatching time
			0	2	4	6	8	
TNAP	0.08	0.09	0.09	0.12	0.16	0.21	0.27	0.35
DNAP	0.02	0.02	0.02	0.03	0.06	0.07	0.09	0.10
RNAP (TNAP-DNAP)	0.06	0.07	0.07	0.09	0.10	0.14	0.18	0.25

At 12 hours after oviposition the amount of RNAP was much higher than DNAP accounting for about 80 per cent of the total nucleic acid P. Both acids showed a slight increase during the pre-

diapause period, but changed little during the diapause. In the post-diapause stage, they both increased rapidly.

Examination of the data of Table 1 reveals that the rate of increase in DNAP is more than that in RNAP, although the absolute amount of increase is larger in RNAP than in DNAP. LU and BODINE (1953) have observed in *Melanoplus* eggs that although the amount of RNAP is much higher than DNAP at the beginning of development, the latter exceeds the former during the post-diapause period. In their case, the increase of DNAP keeps pace with growth, while that of RNAP is slightly irregular, that is, it is much retarded when the rate of growth increases sharply during 10–20 days of pre-diapause and 10-15 days of post-diapause. Basing on these facts, LU and BODINE suggested that RNAP might supply the materials necessary for the DNAP synthesis. In *Bombyx* egg, however, such phenomenon was not observed.

Fractionation of the Acid Soluble P with Barium and Ethanol

Analysis of acid soluble P has shown that at the onset of development, the non-hydrolyzable P accunts for about 50 per cent of acid soluble P and decreases progressively with embryonic growth. This suggests that the non-hydrolyzable P plays a leading role in furnishing the needs of the embryo for phosphorus. With the aim of grasping its chemical nature, fractionation of acid soluble P with barium and ethanol has been undertaken.

The diapause eggs at two different developmental stages were employed as material, the one, at 12 hours after oviposition, and the other, at the onset of post-diapause development (270 days after oviposition). The amount of non-hydrolyzable P was the highest at the former stage and at a fairly low level at the latter stage (see Figure 6).

The method of fractionation is similar to that described by LEPAGE (1949). The experimental results are shown in Table 2. The Table reveals that about 57 per cent of non-hydrolyzable P within the egg 12 hours after oviposition appears in the barium soluble alcohol soluble fraction, and further that the decrease in non-hydrolyzable P during the pre-diapause and diapause period is clearly reflected on the decrease of this fraction. LEPAGE has enumerated 1, 2-propanediolphosphate and aminoethylphosphate as the compound

belonging to the barium soluble alcohol soluble fraction. According to LINDBERG (1946), sea urchin eggs contain a large amount of propanediolphosphate, about 16 per cent of its acid soluble P being occupied by this compound. From this fact he has suggested that this ester might perform an important role in the carbohydrate metabolism of sea urchin eggs.

Table 2. Fractionation of acid soluble P with barium and ethanol at two different stages. Figures represent P in mg. per g. of egg.

	Pre-diapause (12 hours after oviposition)			At the beginning of post-diapause (270 days after oviposition)		
	Barium insol.	Ethanol insol.	Barium sol. Ethanol sol.	Barium insol.	Ethanol insol.	Barium sol. Ethanol sol.
P_0	0.43	0.03	0.00	0.84	0.16	0.00
$P_{10}-P_0$	0.27	0.07	0.00	0.05	0.03	0.00
$P_{180}-P_{10}$	0.10	0.06	0.00	0.02	0.03	0.00
$P_{acid\ sol.}-P_{180}$	0.11	0.42	0.69	0.05	0.43	0.42

In the silk worm egg, the P content of barium soluble alcohol soluble fraction is calculated to be about 30 per cent of acid soluble P at 12 hours after oviposition. Although it is impossible to determine from the present results whether the compound belonging to this fraction is propanediolphosphate or aminoethylphosphate or other unknown compounds, it is possible that the compound or the compounds in question may perform an important role in phosphorus metabolism of the silk worm egg.

In passing, it will be worth while to note that the amount of apparent inorganic P (P_0) belonging to alcohol insoluble fraction increases from 0.03 to 0.16 mg. per g. of egg during the diapause period. This is due to a considerable accumulation of phosphagen during the above period as was described already. In view of the fact that phosphagen appears in alcohol insoluble fraction, this result agrees well with the data obtained from the analysis of acid soluble P within the diapause egg (*cf.* Figure 6).

DISCUSSION

The results presented above indicate that during embryonic development there occurs a marked increase in nucleic acid P and lipid P and a corresponding decrease in acid soluble P. Since the egg of

silk worm is a cleidoic system, the amount of P within each egg should be maintained constant throughout the development. Therefore the P necessary for the synthesis of nucleic acid and phospholipid must be supplied at the expense of acid soluble P. Although the changes occurring in nucleic acid P and acid soluble P during the development of the grasshopper egg are similar to those found in the silk worm egg (Lu and Bodine 1953), the lipid P behaves quite differently, that is, the lipid P of the grasshopper decreases rapidly in the pre-diapause period and remains at a fairly constant level in the diapause and post-diapause phase. This discrepancy between the eggs of the grasshopper and the silk worm will deserve a further investigation.

Another striking difference observed in the diapause eggs of these two kinds of insects is the changes of P compounds during early period. In the silk worm egg, there occurs no marked change in the P of all four fractions examined, except a slight decrease in acid soluble P and a slight increase in nucleic acid P during the first day of development. In the grasshopper egg, on the other hand, nucleic acid P increases steadily, and lipid P and acid soluble P decrease progressively until 20 days of development when the half grown embryo enters diapause, after which they remain nearly constant until the termination of the diapause. In this connection, it should be pointed out that the silk worm enters diapause as an unsegmented embryo at a much earlier stage than the grasshopper. According to Boell (1935), the oxygen uptake of the *Melanoplus* egg increases with embryonic growth during the first 3 weeks of development and then falls off to a level which is maintained throughout the diapause. In the *Bombyx* egg, however, the decline of oxygen consumption comes after the first day of development (Ashbel 1930, 1932, Chino unpublished). Thus the time when the oxygen uptake begins to fall agrees well with the time when the P compounds, mentioned above, reach a constant level. These facts indicate intimate relationship among the embryonic growth, the respiratory activity and the phosphorus metabolism within the egg.

In spite of these, one recalls the fact that various fractions of acid soluble P keep on changing even after the eggs have entered diapause; for example, inorganic P increases up to 30 days, and easily

hydrolyzable and non-hydrolyzable P decrease up to 50 days after oviposition. In this sense, it is not easy to determine the exact time at which diapause sets in *Bombyx* egg, although one can not find any mitotic acitivity in the eggs kept at 25° C. for 10 days after oviposition. Therefore it may be concluded that during the early period of diapause, the liberation of inorganic P from easily hydrolyzable and non-hydrolyzable P proceeds even after the morphogenesis has been completely arrested and the oxygen uptake has fallen to a minimum level.

Now turning again to the problem of P transfer, the most striking feature is probably the rise and fall in inorganic P. This suggests that two kinds of reaction take place; the one, liberating the inorganic P, and the other, incorporating it. It is evident from Figures 2, 4 and 6 that the P compounds liberating inorganic P all belong to the acid soluble fractions including easily and difficulty hydrolyzable and non-hydrolyzable P. Among them, the non-hydrolyzable P seems to play a major role, for it is always found in a high amount at the beginning of development (about 50 per cent of acid soluble P). Non-hydrolyzable P declines more rapidly in the early period before blastokinesis than in the later period. In the early period, the rise of nucleic acid P and lipid P is not so marked and inorganic P increases steadily, while in the later period there occurs a sharp increase of nucleic acid P and lipid P, accompanied by a striking decrease of inorganic P. From these observations, it seems probable that inorganic P liberated from non-hydrolyzable P is incorporated into nucleic acid and lipid P. However, it is unlikely that inorganic P is liberated directly from non-hydrolyzable P, or it is incorporated directly into nucleic acid and lipid P. Probably some organic ester acts as an intermediate in the above reactions. It might also be possible to imagine that P is delivered from non-hydrolyzable P into nucleic and lipid P without the liberation of inorganic P. At any rate, it may be assumed that the inorganic P increases when its liberation from various acid soluble P, especially from non-hydrolyzable P, exceeds its incorporation into nucleic and lipid P, and it decreases under a reverse condition. In order to confirm this assumption, the following experiment has been carried out. Non diapause eggs at 60 and 84 hours of development were respectively ex-

posed to N_2 gas for 24 hours.* Such eggs were analysed for various P compounds before and after the treatment and compared with aerobic controls. The results given in Tables 3 and 4 show that anaerobiosis induces a marked increase in inorganic P and a considerable decrease in labile and non-hydrolyzable P, and further that it completely suppresses the increase of nucleic acid and lipid P.

The behavior of inorganic P under aerobic condition is quite different between these two stages at which the eggs were exposed to nitrogen; inorganic P increasing at 60 hours and decreasing at 84

Table 3. Change in the P component of non-diapause egg at 60 hours after 24 hours of anaerobiosis. Each figure represents P in mg. per g. of egg.

Component	At beginning of experiment	After 24 hours		Increase (+), decrease (−) during 24 hours	
		Air	Nitrogen	Air	Nitrogen
P_0	0.83	0.88	1.09	+0.05	+0.26
Inorganic P	0.73	0.77	0.99	+0.04	+0.26
$P_{10}-P_0$	0.35	0.32	0.20	−0.03	−0.15
$P_{180}-P_{10}$	0.22	0.20	0.24	−0.02	+0.02
$P_{acid\ sol.}-P_{180}$	1.18	1.09	1.05	−0.09	−0.13
Acid sol. P	2.58	2.49	2.58	−0.09	0
Lipid P	0.37	0.40	0.37	+0.03	0
Nucleic acid P	0.26	0.32	0.26	+0.06	0
Protein P	0.12	0.12	0.12	0	0

Table 4. Change in the P component of non-diapause egg at 84 hours after 24 hours of anaerobiosis. Each figure represents P in mg. per g. of egg.

Component	At beginning of experiment	After 24 hours		Increase (+), decrease (−) during 24 hours	
		Air	Nitrogen	Air	Nitrogen
P_0	0.88	0.83	1.19	−0.05	+0.31
Inorganic P	0.77	0.71	1.08	−0.06	+0.31
$P_{10}-P_0$	0.32	0.30	0.16	−0.02	−0.16
$P_{180}-P_{10}$	0.20	0.17	0.23	−0.03	+0.03
$P_{acid\ sol.}-P_{180}$	1.09	1.07	0.91	−0.02	−0.18
Acid sol. P	2.49	2.37	2.49	−0.12	0
Lipid P	0.40	0.45	0.40	+0.05	0
Nucleic acid P	0.32	0.39	0.32	+0.07	0
Protein P	0.12	0.12	0.12	0	0

* Twenty four hours anaerobiosis retards the development considerably, so that the total embryonic period was prolonged for about two days.

hours. Nevertheless the effect of anaerobiosis upon the liberation of inorganic P shows no essential difference as can be seen in the tables.

BARTH *et al.* (1947) have observed that when frog eggs are exposed to N_2 gas at gastrula stage, the inorganic P increases considerably, and the labile P decreases abruptly, and the two approximately coincide in time. In the present experiment, however, the decrease in labile P is only about half of the increase in inorganic P, and the decrease of non-hydrolyzable P makes up for this difference. These facts also support the view, mentioned previously, that non-hydrolyzable P performs an important role for phosphorus metabolism within the silk worm egg.

Fractionations of acid soluble P with barium and ethanol have indicated that the decrease in non-hydrolyzable P during growth occurs mainly in the barium soluble-alcohol soluble fraction. The identification of the compounds belonging to this fraction will be undertaken in the near future.

SUMMARY

1) The changes of various phosphorus (P) compounds during embryonic development has been studied in three types of the silk worm egg, that is, non-diapause egg, artifitial non-diapause egg and diapause egg.

2) In the non-diapause or artifitial non-diapause egg, acid soluble P decreases steadily with the development, while total nucleic acid P and lipid P increase progressively. Protein P is at all times very low and shows no marked change throughout the development.

In the diapause egg, no change occurs in the P of the above four fractions throughout the pre-diapause and diapause periods, except a slight decrease in acid soluble P and a similar increase in total nucleic acid P during the first day of the development. The P changes during the post-diapause phase are similar to those found in the non-diapause egg.

3) Both ribonucleic acid P (RNAP) and desoxyribonucleic acid P (DNAP) increase with development. DNAP increases more rapidly than RNAP, although the latter exceeds the former in the absolute amount of the increase.

4) A most striking feature of the P changes in acid soluble fractions is the rise and fall in inorganic P and the steady decline of non-hydrolyzable P. Easily and difficultly hydrolyzable P decrease to a minimum value about half way through the development, then the former fraction increases gradually up to the time of hatching, while the latter changes little. In diapause egg, the increase of inorganic P and the decrease of easily and difficultly hydrolyzable and non-hydrolyzable P continue for a while even after the egg has entered diapause.

5) Fractionation of acid soluble P with barium and ethanol indicates that at the beginning of development about 57 per cent of non-hydrolyzable P appears in the barium soluble alcohol soluble fraction, and that the decrease of the non-hydrolyzable P through the development occurs mainly also in this fraction.

6) The amount of phosphagen, given by the difference between true inorganic P and apparent inorganic P, remains nearly constant throughout the development in non-diapause and artificial non-diapause egg, while it increases considerably in the diapause egg during the early period of diapause and it decreases abruptly during the first 3 days of post-diapause development.

7) The application of anaerobic condition results in a marked increase of inorganic P with corresponding decrease of easily hydrolyzable and non-hydrolyzable P and suppresses the increase of nucleic acid P and lipid P completely.

8) From the data obtained, the transference of P in the egg, especially the role of non-hydrolyzable P, has been discussed.

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ON THE MORPHOLOGY OF THE MITOTIC APPARATUS ISOLATED FROM ECHINODERM EGGS

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In a previous paper, a technique was reported for obtaining the mitotic apparatus in isolated condition from alcohol-fixed eggs of the sea urchin, *Strongylocentrotus franciscanus* (MAZIA and DAN 1952). In the present paper, the same technique was applied to different kinds of sea urchins, partly for the purpose of comparison and partly to carry out more detailed study of various stages in the formation of the mitotic spindle and its asters.

MATERIALS AND METHODS

Eggs of the Japanese sea urchins, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* were used. Since both these species gave essentially the same results, the descriptions given here will be based mainly on the latter, although some fragmentary observations made on the Woods Hole sea urchin, *Arbacia punctulata* and the Tokyo Bay starfish, *Asterias amurensis*, have also been included.

Eggs were fertilized by the standard technique and were cultured until the desired developmental stage had been reached. Special care was taken to rear them sparsely to insure a uniform pace of development, and they were concentrated by centrifugation before fixation by cold alcohol. When necessary, the fertilization membrane was removed. For this purpose cysteine treatment of unfertilized eggs was practiced in *Pseudocentrotus* (RUNNSTRÖM, MONNÉ and BROMAN 1943), while in *Hemicentrotus*, the eggs were shaken in a test tube about one minute after insemination. However, in the majority of cases, the membranes were intentionally left intact because, al-

though the mitotic apparatus can be liberated from the cell, the fertilization membranes survive the detergent action, thus giving an assurance that the shape of the liberated structure has not been distorted by external mechanical forces.

At the desired stage, the eggs were fixed in a fairly large amount of 30% ethyl alcohol kept at about $-10°$ C. If the amount of alcohol used is small, the heat of dilution of the alcohol on the addition of the egg suspension raises the temperature of the mixture sufficiently to spoil the result. For the isolation, the H_2O_2-Duponol D method was followed. MAZIA (1953) has reported that digitonin treatment can be substituted for the above method. Although the latter is presumably a more desirable technique because of the mildness of the action of the reagent, this was not practiced in the present work. Certainly the use of H_2O_2 is cumbersome because of the evolution of a large amount of O_2, since the activity of the egg catalase is preserved in the cold alcohol. In our experience, monoiodoacetic acid can be used to inhibit the catalase activity. However, upon doing so, it was accidentally found that when the eggs are treated by monoiodoacetic acid, the H_2O_2 treatment could be skipped entirely; the reason for this, however, is not clear.

After the isolation, it is necessary to use a phase contrast microscope to make out details of the structure, as the isolated mitotic apparatus is highly swollen in the detergent.

RESULTS

I. Sea Urchins

Pre-cleavage stages

a) Pronucleus: The isolation of the egg pronucleus is the easiest of all and rather casual or even rough treatment of the cells by the detergent gives a large number of egg pronuclei which appear reticular inside (Fig. 1*a*).

b) Monaster: While the sperm heads remain compact immediately following their penetration into the eggs, it is impossible to identify them after isolation. However, the application of this technique a few minutes later gives monasters with sperm pronuclei at their centers. After a few more minutes, when the egg pronucleus

penetrates into the monastral zone just before syngamy, the two pronuclei can be isolated imbedded in a common aster, regardless of whether the egg- and sperm-pronuclei are in contact or are still separated by some distance. Since no special connecting structure can be found between the two pronuclei in this stage, the two pronuclei must be held together simply because they are included in the same astral structure. This is likely since in *Spisula* (Mollusca), in which no monaster is formed, the two pronuclei fall apart if they are isolated before they establish contact.

c) Streak stage: The streak stage of sea urchins is, in a sense, a very problematical stage. Concerning the shape of the streak, MONNÉ (1944) thinks that it is a disc composed of a special cytoplasm which is characterised by a negative birefringence along its long axis, in contrast to the positive birefringence of the spindle and the astral rays. A synkaryon is situated at the center of the disc. As is well known, the streak is perceived only when the cells are observed from a certain angle and MONNÉ interpreted this as being due to the fact that the streak is apparent only when the disc is looked at along its flat surface.

Although the result of a birefringence study conducted by INOUÉ and DAN (1951) agrees with MONNÉ's findings, the present authors feel that such data alone do not necessarily constitute adequate basis for definitely assigning a disc shape to the streak. Consequently, they hoped that the isolation technique might settle this point. However, it turned out to be otherwise, and the shape of the astral systems isolated from the monaster stage and from the streak stage did not differ enough to distinguish between them, except for the fact that in the latter stage, the center has divided into two (Figs. 2, 3). This may be because what was isolated was a whole astral system and the streak inside may have become unrecognizable by the treatment. At any rate, the streak stage is a stage at which the center divides.

Cleavage stages

d) Prophase: As was reported in *Stroyngylocentrotus franciscanus*, the nucleus of *Hemicentrotus* in the late prophase which looks coarsely reticular inside is always associated with two small

asters on opposite sides of it (Fig. 5); in *Arbacia*, the asters develop to a fairly large size before the nuclear membrane ruptures (Fig. 1 *c*). Such early asters are provided with centers which appear as dark dots with a dark contrast objective. The centrioles are usually single, but sometimes several small dots are seen instead.

e) Prometaphase: The transition from the prophase to the metaphase is the most difficult stage to study, chiefly because only a few cases have been met with. This is presumably due to the short duration of this stage. In one case observed in *Arbacia* (Fig. 4), the nuclear membrane seems to be still intact, appearing pushed in at opposite sides by the lengthening astral rays. The situation is very much like that represented in WILSON's figure (WILSON 1895, Pl. VI, Fig. 24 and Text-Fig. 13, *B*). In a few other instances in *Hemicentrotus*, although the nuclear membrane has been lost, the spindle is obviously not complete. It is called incomplete in the sense that no continuous fibers from pole to pole can be seen and two, more or less separate, half spindles are recognizable (Figs. 6, 7). Whether or not this state can be looked upon as the prometaphase cannot be said with certainty, since the present method is not particularly suited for the study of chromosomes, as will be explained in the following section. At any rate, one thing which is certain is that the asters at this stage are definitely larger than those of the prophase stage.

f) Metaphase: The isolated metaphase structure is composed of a pair of asters, a pair of centers and the spindle. But almost invariably the chromosomes are not visible (Figs. 1 *d*, 8, 9, 14). This apparent absence of chromosomes was observed with the sea urchins of California as well as of Misaki. When the previous paper was written (MAZIA and DAN 1952), the writers were not certain whether the absence of chromosomes was due to a real loss of the chromosomes during the isolating procedure or was simply an optical effect. However, it is now certain that the latter situation is true for two reasons. (1) MAZIA (1953) finds that in digitonin preparations, chromosomes are clearly seen on the equatorial plate. (2) In *Hemicentrotus*, if the astral rays are dissolved off more than usual either by reducing the H_2O_2 treatment or by prolonging the detergent treatment, chromosomes become definitely visible (Fig. 12). This suggests

that with our standard method, the chromosomes are hidden under the median crossing rays, since with phase contrast the surface images predominate and conceal the interior.

The fact that the length of the rays on the isolated asters can be made shorter by decreasing the H_2O_2 concentration or by strengthening the detergent treatment shows that the shape of the isolated aster is a relative thing. For this reason, when the size of the aster is compared between different stages, the isolation technique should be kept strictly identical. Digitonin is believed to be a milder detergent and it gives longer astral rays than are found in preparations treated with Duponol D.

g) Anaphase and telophase: Some difficulty in observing the chromosomes because of the covering rays still persists through the anaphase and until the telophase (Figs. 1*e*, 10, 15). But once the crossing rays are removed, the behavior of the underlying nuclear elements at these stages accords with orthodox cytological knowledge (movement of chromosomes, lengthening of the interzone, etc.) (Fig. 13).

There are, however, two points which the isolation technique enables us to determine with certainty. (1) As long as the identical technique is used, the size of the aster steadily grows through metaphase until the telophase or even until the early restitution stage, at which time the aster attains its maximum size. This agrees with our experience with living sea urchin eggs. (2) Previous workers have given only very vague descriptions of the interzonal region; with the present technique, it was found to be a structure of a definite integrity, extending between the two daughter nuclei.

h) Cytoplasmic division: The structure isolated at this stage is particularly significant. As is shown in Figs. 11 and 16, a long and slender but definite rod is found connecting the two nuclei and their accompanying asters. The one shown in Fig. 11 is particularly surprising because it was isolated from an egg which was almost completing cleavage. That Fig. 11 is a very late stage is also indicated by the disappearing asters. This rod must certainly be the remnant of the interzone which is often believed to have disappeared by this late stage. In a microphotograph shown previously in connection with *Strongylocentrotus* (Mazia and Dan 1952, Fig. 10), this

rod was of an elongated spindle shape, but those obtained in *Hemicentrotus* and *Arbacia* are bars of uniform width. To some extent this difference may be accounted for by a difference in stage, the former being earlier than the latter.

Furthermore, this connecting structure is very frequently provided with a row of granules strictly bisecting the bar so that these granules might be mistaken for chromosomes on the equatorial plate. But there is absolutely no possibility of a mistake because for one thing, the row extends beyond the contour of the spindle, and for another, the chromosomes are already incorporated in the daughter nuclei at this stage.

Later cleavages

i) Second and third cleavages: Practically the same process is repeated for the second and third cleavages. For these and later cleavages, it is convenient to leave the fertilization membranes intact, since they hold each complete set of mitotic apparatuses together. Figures 17 and 18 show the mitotic apparatus of eggs going from the 4-cell to the 8-cell stage, while Figure 19 represents the next division.

j) Fourth cleavage: The fourth cleavage is of special interest because this division gives rise to eight mesomeres, four macromeres and four micromeres in sea urchin larvae. In other words, the four animal cells of the 8-cell stage divide equally, serving as a natural control, while the remaining four vegetal cells cleave unequally. The application of the isolation technique to the 8-cell stage which is about to enter division releases eight mitotic apparatuses. Until early metaphase, there is no way to distinguish the mitotic apparatuses of the animal and vegetal blastomeres, both groups having small, equally sized asters (Fig. 19). By mid-metaphase, however, four of the eight spindles acquire asters of an equal size at the two ends (Fig. 20 an) while the other four spindles obtain asters of different sizes and shapes at the opposite ends of the spindle (Fig. 20 veg). In the vegetal cells, the aster which is to form a macromere is of a spherical shape as usual (Fig. 20 veg upper poles) but the aster at the micromere ends of the spindle is truncate or flat-topped (lower poles).

II. Starfish

The same method of isolation has been used with similar success on the eggs of the Tokyo Bay starfish, *Asterias amurensis*. In the unfertilized condition, germinal vesicles with conspicuous nucleoli are isolated and later very tiny maturation spindles can also be set free. However, it was impossible to photograph the polar spindles because of continuous Brownian movement of the tiny mitotic elements.

Metaphase and anaphase apparatuses look almost the same as those of sea urchins but the course of changes was not followed as the material available was limited.

Discussion

Meaning of cold alcohol: From the beginning, the main reason for choosing 30% ethyl alcohol at $-10°$ C. was that it would kill (or fix) the cells with a minimum denaturation of their proteins. This expectation turns out to be true. If cold alcohol of higher concentrations are used, the cytoplasm is strongly coagulated so that later isolation becomes extremely difficult or impossible. If eggs are treated with cold 20% alcohol, the cells are not fixed immediately as shown by the fact that they are able to recover if the treatment is stopped after the first several minutes. This means that instead of acting as a fixative, 20% cold alcohol acts simply as an adverse condition and eventually causes cytolysis.

On the other hand, if 30% ethyl alcohol is applied at room temperature, its effect is more deleterious than 20% at room temperature, the eggs swelling immediately and forming vacuoles. According to Lee (1928), 30% alcohol kept at room temperature used to be called "Ranvier's alcohol" and was employed as a maceration medium rather than as a fixative.

If 30% alcohol is made up with sea water and cooled, eggs fixed in this mixture cannot be used for isolation. This indicates that the low salt content of the alcohol solution is another important factor.

On the contrary, 30% alcohol made up with distilled water and cooled to a sub-zero temperature can be a fine fixative even for ordinary cytological purposes, as tested by sectioning and staining onion root tips and sea urchin eggs (Figs. 21, 22). From the above, it

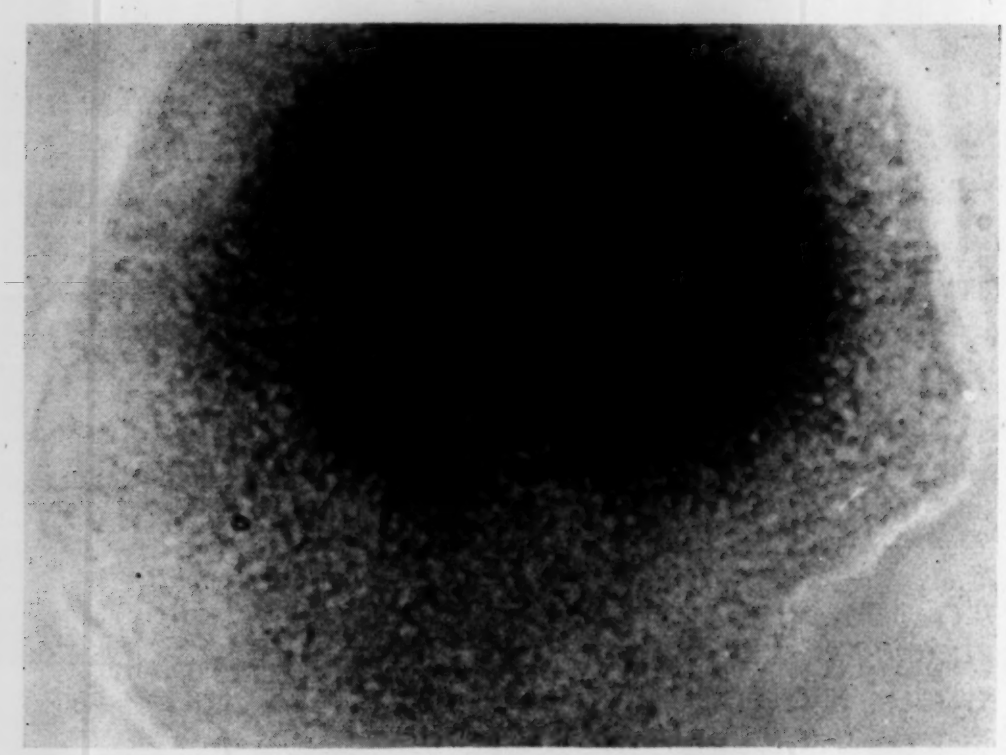


Fig. 21

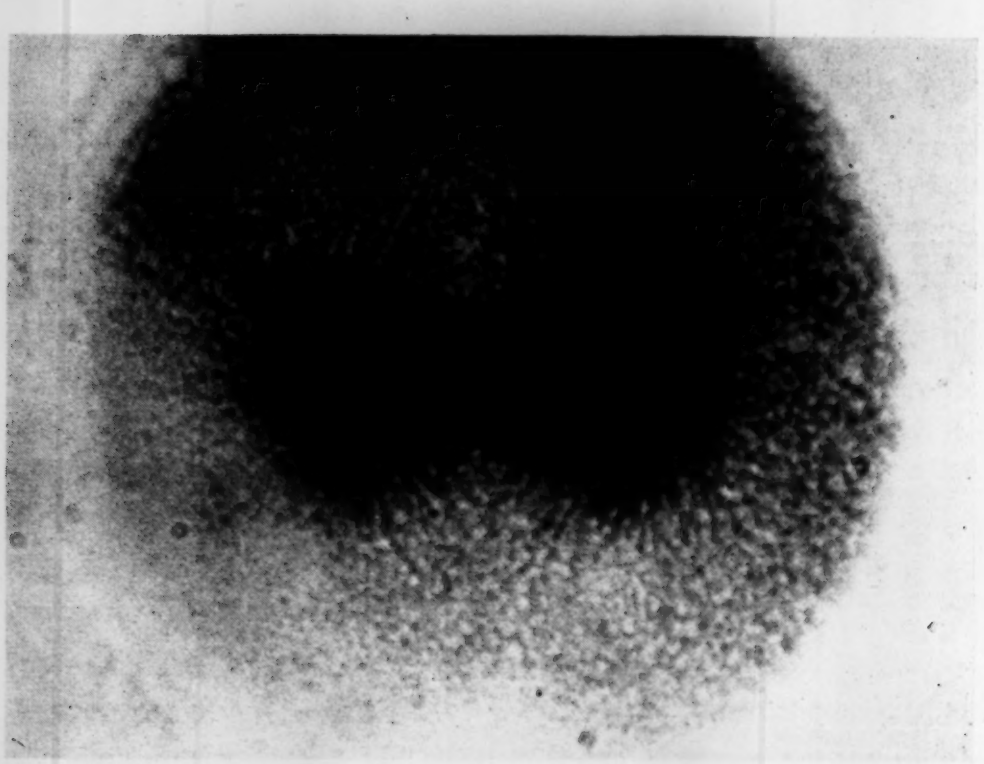


Fig. 22

Fig. 21, Fig. 22. Sections of the eggs of *Hemicentrotus pulcherrimus* fixed in cold 30% ethyl alcohol and stained with HEIDENHAIN haematoxylin, showing that the stained parts correspond with isolated structures. (Prepared by K. KAWAMURA)

seems possible that the beneficial effect of coldness may lie in the paralysis of enzymes when they are more or less chaotically liberated on the death of the cell. But once this volley of enzymes rendered ineffective either by diffusion out of the cell (as in the case of catalase) or by impairment of the cell organization, the mitotic apparatus left by itself has an amazing stability, as shown in this paper.

At the first glance, such stability appears quite contrary to our experience with the living mitotic apparatus, which has been found to be very sensitive and unstable, being immediately abolished by any adverse condition or rough handling. Even under normal conditions the mitotic apparatus is destined to disassemble itself within 10–15 minutes, when each division is complete. From our present standpoint, the formation and disappearance of the mitotic apparatus cannot be considered to be simple events. The cell must be in a state of vigorous activity, mobilising a series of enzymes for the formation of the mitotic apparatus, to be followed immediately by another volley of enzymes for its removal.

The aster: In the foregoing paragraphs it was mentioned that the size of isolated asters is a relative thing, because the final length of the rays depends on the degree of stiffening by H_2O_2 and the degree of later dissolution by the detergent. But since the isolation itself can only be achieved by erosion of the cell from the periphery by the detergent, no matter how carefully the isolation is practiced, by the time the isolated products are "clean", a considerable part of the periphery is gone and therefore the isolated asters are much smaller than their original size in the intact cell.

This may seem to endanger DAN's previous contention that the tips of the astral rays are anchored to the cell periphery (DAN 1943). However, another and probably a more direct reason for the shortness of the isolated rays lies in the fact that cold alcohol fails to fix the peripheral of the rays from the beginning as can be judged by the section of alcohol-fixed cells (Figs. 21, 22). Why, then, the peripheral rays which are visible in the living condition and are preserved in other fixatives are lost on alcohol fixation is impossible to answer at present.

SCHMIDT (1937) and MITCHISON and SWANN (1955) have expressed doubt as to whether the rays ever reach the cell periphery in the

living condition. SCHMIDT's objection is based on the fact that in *Cerebratulus* eggs the birefringence of the rays cannot be detected on the periphery. INOUÉ and DAN (1951, p. 433) have pointed out the inadequacy of the basis of SCHMIDT's argument, since in *Cerebratulus*, there is a high possibility of cancellation of the radially positive birefringence of the rays by the radially negative birefringence of the cortex. (The *Cerebratulus* egg cortex is exceptional among marine ova in having a radially negative birefringence.) In *Clypeaster*, it is not difficult to trace the rays to the cortex by their double refraction (see INOUÉ and DAN 1951, Plate 2, Fig. 10).

The idea of MITCHISON and SWANN was derived from the easiness of movement of the asters within the cytoplasm. However, HIRAMOTO (unpublished observation) has displaced the asters with a micropipette and finds that the rays are bent or stretched with their tips always remaining in the cortex.

In connection with the aster, by far the most surprising situation is the difference found in the shapes of the aster of unequally cleaving cells. GRAY (1931) and DAN (1943) anticipated that when the two asters were spheres of different diameters, the resulting daughter cells would be different in size. It might have been expected that two asters of quasi-equal diameter, but one perfectly spherical and the other truncate, as found here, would also give rise to unequal blastomeres. Still more exciting is the fact that the same circumstance is also found in unequally cleaving molluscan egg, as was reported previously (DAN, ITO and MAZIA 1952).

The connecting bar and the bisecting row of granules: A question will immediately be raised about the nature of the bar and the row of granules which are isolated from the late division stage. The former is obviously the remnant of the interzone of the spindle. This notion may be difficult to harmonize with findings among plant cells, since if a spindle remnant ever exists in plant cells, it must have changed into the phragmoplast by this stage. In animal cells, on the other hand, the presence of this structure was already made evident by its birefringence (INOUÉ and DAN 1951). But in the literature, although there are occasional accounts of this structure as the interzone immediately after chromosome separation and as the mid-body in the very late stage, hardly any reports are available on

the intermediate conditions. The present result has unequivocally demonstrated its continuous existence throughout these periods.

With regard to the row of granules running perpendicularly to the spindle axis, two more or less similar structures have been reported. One is the precursor of the mid-body on the telophase spindle. The other is the diasteme in the ovum, which is a differentiated sheet of cytoplasm bisecting the cell and delineating the cleavage furrow which is about to cut in (MOTOMURA 1950, SELMAN and WADDINGTON 1955). However, in the former, unlike the present case, the row of granules never extends beyond the border of the interzone, while in the case of the diasteme, no opinion has been ventured concerning its relationship to the spindle.

Purely with respect to its topographic position, this row of granules corresponds more closely to the phragmoplasm of plant cells. When the furrow constriction nearly pinches off the incipient blastomeres, this sheet of granules comes to lie within the narrow connecting stalk in such a direction as to partition the space within the stalk. As a matter of fact, there is one physiological indication of such septum formation in living sea urchin eggs. If dividing eggs are put into a hypotonic medium before the furrow constriction is complete, the swelling process abolishes the furrow and the incipient cells return to the spherical shape; while if the transferrence is made as soon as complete separation is established, the furrow is preserved and the two blastomeres swell separately.

Association of the nuclear and cytoplasmic elements in the mitotic apparatus: Another striking feature brought out by the isolation method is the fact that the entire mitotic apparatus can be removed from the cell *in toto*. As is so well known, such a mitotic apparatus contains nuclear as well as cytoplasmic elements. Thinking of the dual nature of the apparatus, the stage in which such an association is least expected is the prophase when the nucleus is still retaining an intact membrane. But even here the two small asters are always stuck to the nuclear membrane to such an extent that they not only survive elution by the detergent but require vigorous pipetting to separate some of them mechanically.

Although nothing is known about the molecular nature of this association, the recognition of such a firm union between the nuclear

and cytoplasmic elements in the mitotic apparatus might lead to a new view of the make-up of this apparatus.

Summary

1) Mitotic apparatuses were isolated from echinoderm eggs fixed in 30% cold alcohol followed by treatment with H_2O_2 and Duponol D.

2) The sea urchins, *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus* and *Arbacia punctulata* and the starfish, *Asterias amurensis* gave essentially the same results, which are as follows:

3) At any stage, the mitotic apparatus is isolated as a complete ensemble of the asters, centers, spindle and chromosomes.

4) The stages from which the apparatuses were isolated can be identified by their morphology according to orthodox cytological criteria.

5) The size of the aster steadily grows from the prophase to the early restitution stage, after which the aster begins to fade away. In unequal cleavage, although the two asters on a spindle remain equal in size until the early metaphase, by the mid-metaphase, the one which will form the larger cell is spherical, while the other aster which is to go into the smaller cell becomes truncate on the distal side.

6) After the metaphase, the interzone persists until after the completion of the cleavage. At a very late stage of furrowing, the interzone is bisected by a perpendicular row of granules which extends a short distance into the surrounding cytoplasm.

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Explanations of figures

Plate 3. Mitotic apparatuses of *Arbacia punctulata.*

Fig. 1 *a.* Egg pronucleus.
b. Streak stage.
c. Prophase.
d. Metaphase.
e. Anaphase.

Fig. 2. Early streak stage.

Fig. 3. Fully formed streak stage.

Fig. 4. Prometaphase.

Plate 4. Mitotic apparatuses of *Hemicentrotus pulcherrimus.*

Fig. 5. Prophase stage with a pair of small asters attached to the nuclear membrane.

Fig. 6, Fig. 7. Prometaphase, in which the spindle is not yet complete and the two separate halves are recognizable.

Fig. 8, Fig. 9. Metaphase. Chromosomes are not visible.

Fig. 10. Anaphase or early telophase.

Fig. 11. Isolated structure from an egg which was almost completing its cleavage, showing a pair of disintegrating asters, a pair of daughter nuclei, a connecting bar with a bisecting row of granules. Fertilization membrane was left intact.

Fig. 12, Fig. 13. Mitotic apparatuses from which the crossing rays are dissolved off, showing the chromosomes and centers. These stages correspond respectively to those shown in Fig. 9 and Fig. 10. The scale indicated is for Figs. 5–13.

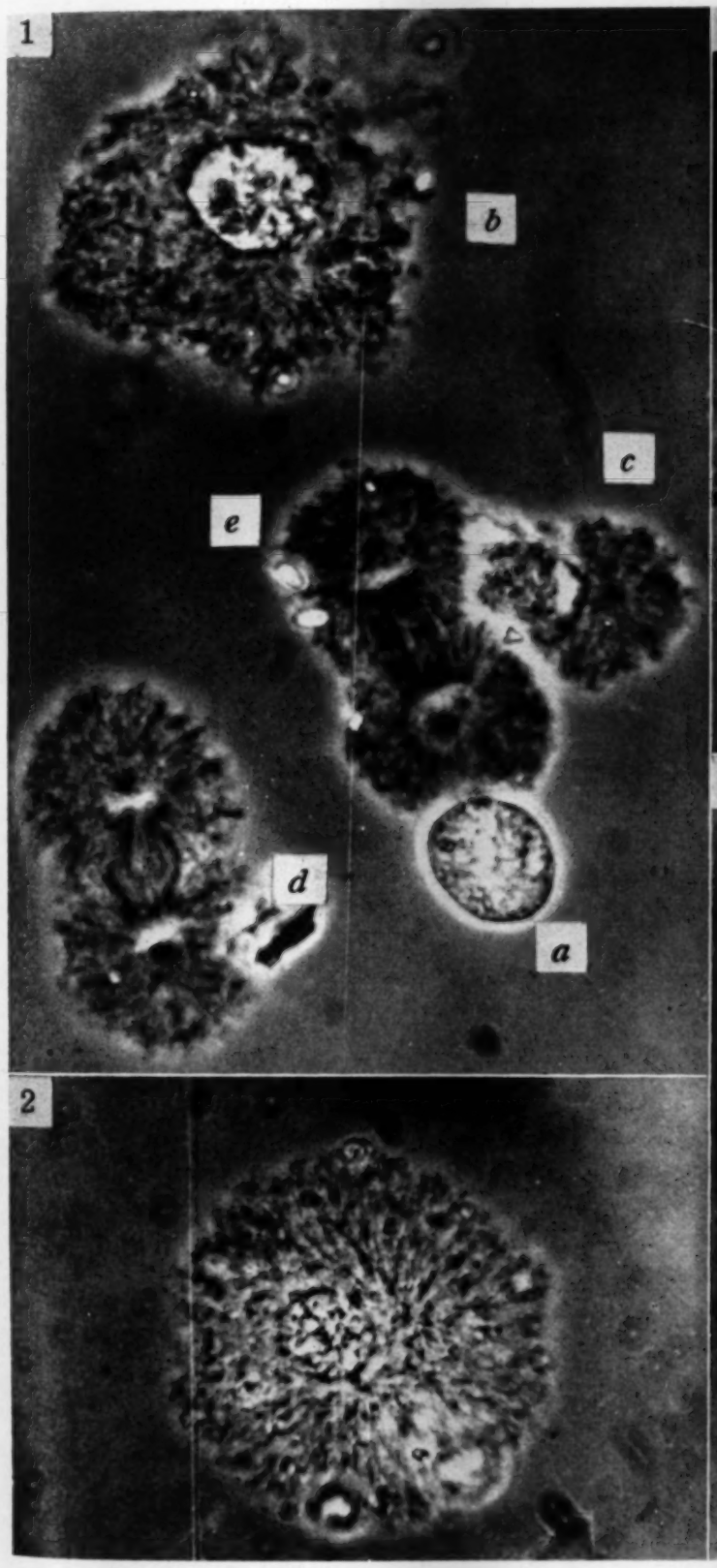
Fig. 14, Fig. 15, Fig. 16. Enlarged pictures of metaphase, telophase and restitution nuclear stages; see scale given at left. Fig. 16 shows the connecting bar and the centers which have divided.

Plate 5. *Hemicentrotus pulcherrimus.*

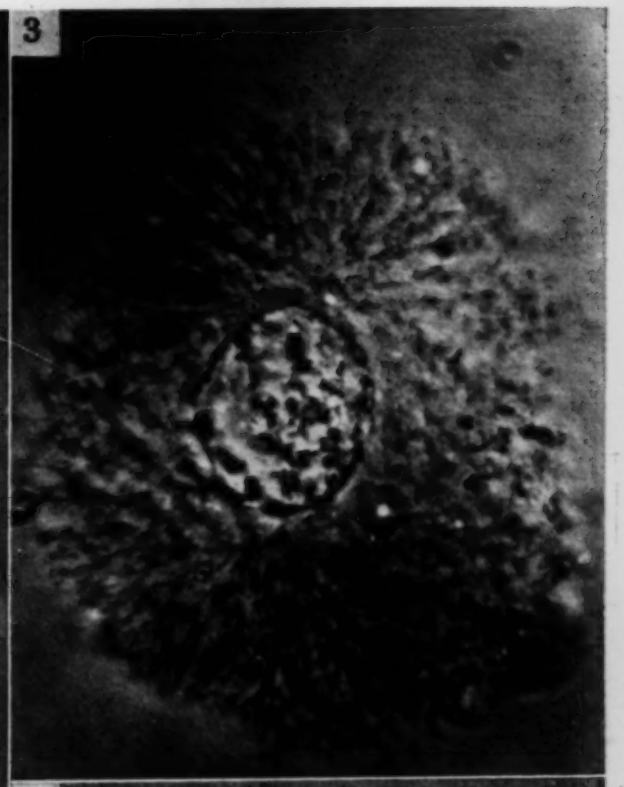
Fig. 17, Fig. 18. Four sets of spindles from the 4-cell stage set free within the fertilization membrane; Fig. 17 at early metaphase and Fig. 18 at telophase. Scale shown at right.

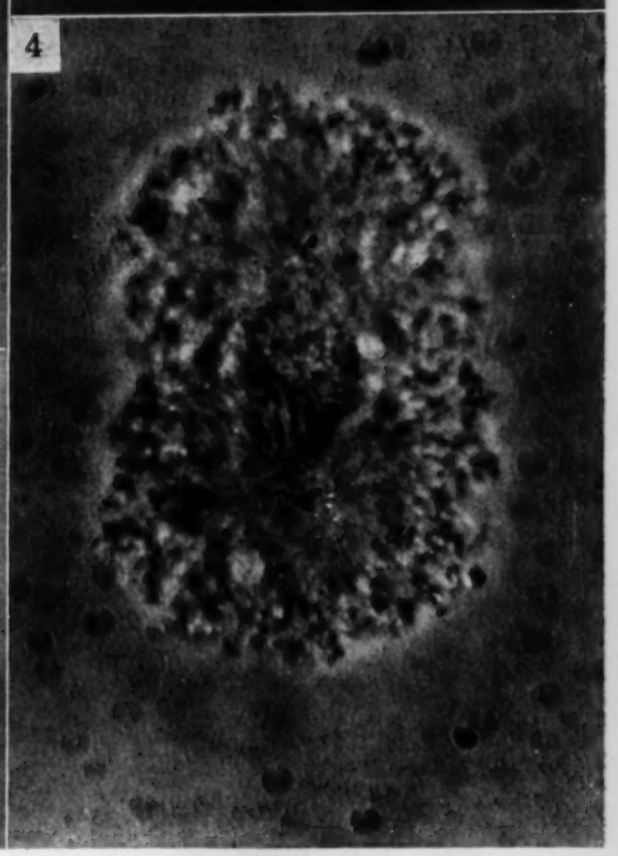
Fig. 19. Eight prophase nuclei from 8–16 cell stage.

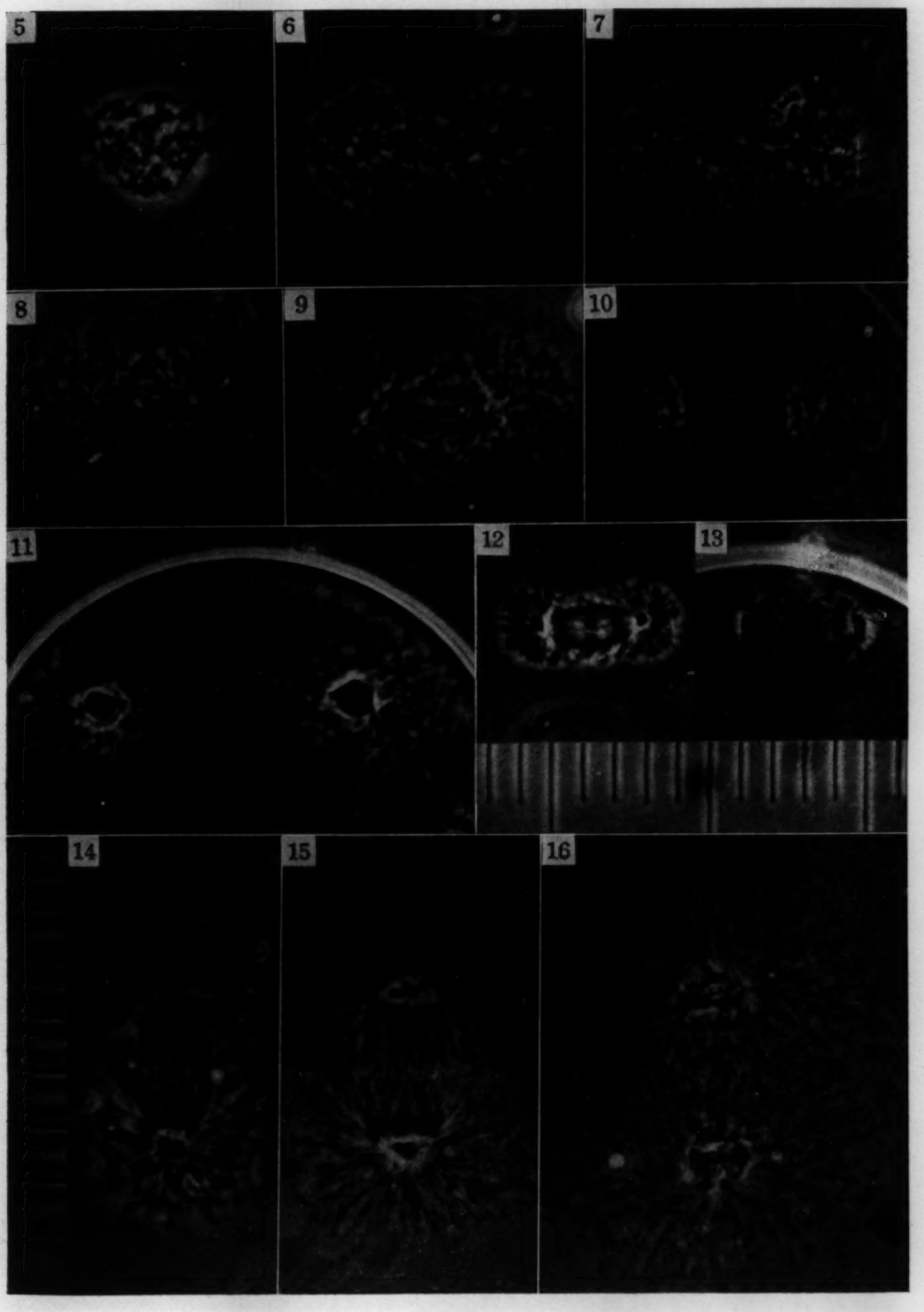
Fig. 20. Sets similar to above at full metaphase. Fertilization membrane was ruptured just before photographing. The four spindles in the above row are obviously four animal cells which are going to produce eight mesomeres, while the lower four with unequally sized asters are the vegetal four cells. The pole with a larger aster is the macromere pole and the one with a smaller, truncate aster is the micromere pole.


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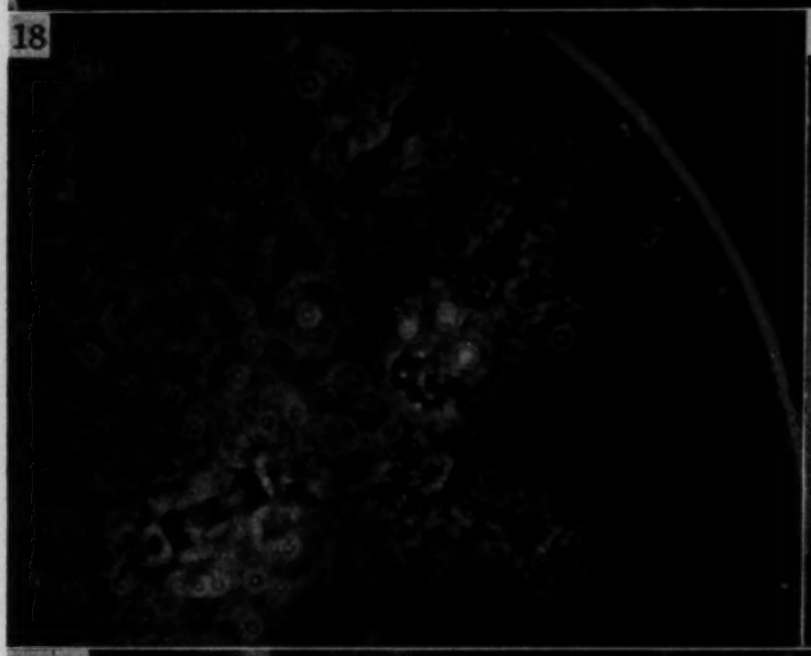

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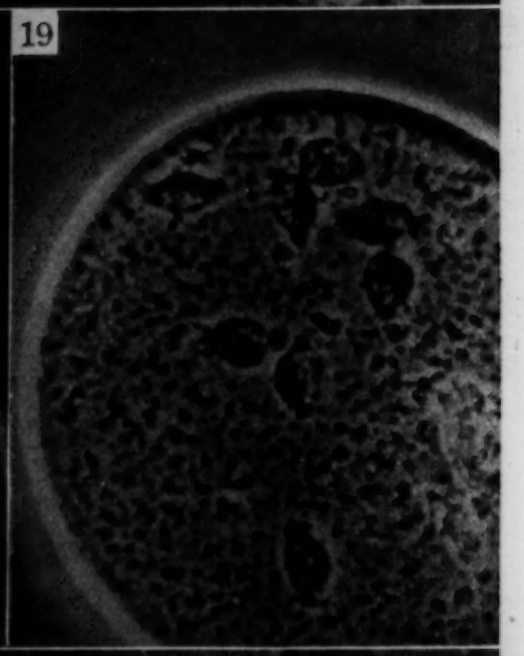
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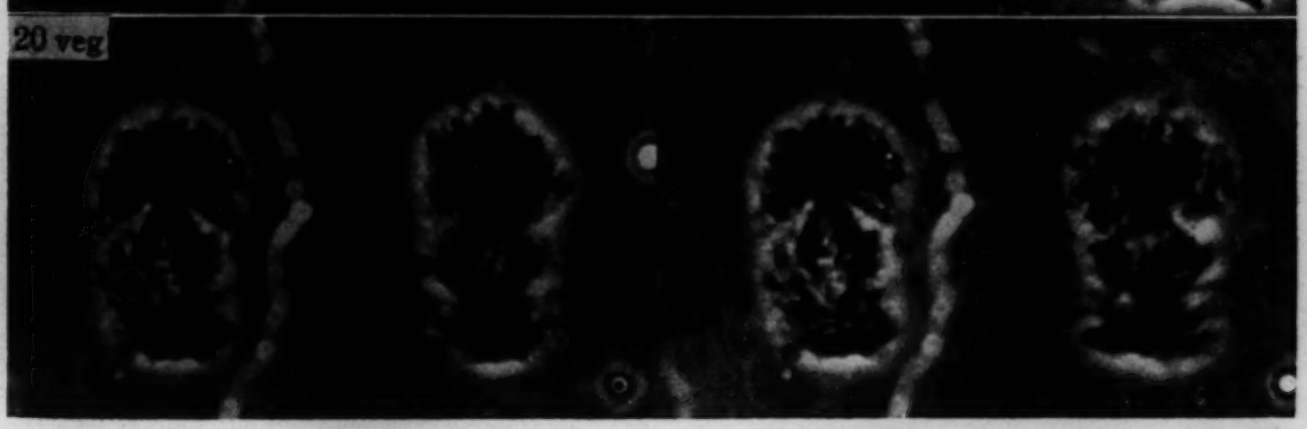
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EMBRYOLOGIA, Vol. 3, No. 3, pp. 201-219 (January, 1957)

ÜBER INDUKTIONSWIRKUNGEN VON BAKTERIENAGGLOMERATEN IM *TRITURUS*-KEIM UND DIE CHEMISCHE NATUR DES WIRKSAMEN STOFFES

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EINLEITUNG

Außer den Induktoren der Normogenese besitzen zahlreiche abnorme Agenzien die Fähigkeit, das präsumptive Ektoderm der Urodelengastrula zur Ausbildung von Induktionsgebilden zu veranlassen. Besonders tierische Gewebe aus fast allen Tierstämmen, unter diesen im stärksten Maße die Wirbeltiere, hatten sich als induktionsfähig erwiesen (u.a. HOLTFRETER, 1935 a). Eigenartigerweise zeigten im Gegensatz dazu Implantate pflanzlicher Herkunft keine oder nur äußerst schwache und vereinzelte Induktionsleistungen: Pflanzliche Reservestoffe (Stärke) erwiesen sich als völlig inaktiv (HOLTFRETER, *op.c.*). Auch frische Pflanzenteile aller Art, selbst Meristeme, in welchen lebhafte Stoffsynthesen stattfinden, waren nach den verschiedensten Aufbereitungs- und Applikationsweisen entweder unwirksam oder riefen lediglich vereinzelte Epidermisverdickungen mit etwas Pigment hervor (TOIVONEN, 1938). Eine neuroide oder gar neurale Induktionsfähigkeit von Birkenkambium-Brei, wie sie RAGOSINA (1938) festzustellen glaubte, ist sehr in Frage zu stellen, da eine exakte histologische Analyse wegen der viel zu frühzeitigen Fixierung nicht möglich war (vgl. hierzu auch die Besprechung von RAVEN, 1938). Es schien daher eher, daß es sich bei den induktionswirksamen Stoffen um solche handelt, die für die tierischen Zellen charakteristisch sind (HOLTFRETER, *op.c.*).

In diesem Zusammenhang war es von Interesse, auch einmal Vertreter des dritten Organismenreiches, der Bakterien, auf Induktions-

fähigkeiten zu prüfen. Die besonderen stofflichen Verhältnisse bei den Bakterien ließen hierbei auf aufschlußreiche Ergebnisse hoffen: Unter allen Organismen sind die Bakterien die bei weitem eiweiß- und nucleinsäurereichsten. Gerade diese Stoffgruppen werden nämlich von vielen Untersuchern in Zusammenhang mit den Induktionsvorgängen gebracht. Während der logarithmischen Wachstumsphase kann die Trockensubstanz der Bakterien bis zu 74% (im Mittel 57%) aus Eiweißen und bis zu 28% (im Mittel 15%) aus Nucleinsäuren bestehen (BELOZERSKY, 1947). Die Nucleinsäuren bestehen zu 70–75% aus Ribonucleinsäure (VENDRELY, 1946).

Außer diesem Grund lag noch ein anderer vor, der ein Bakterienagglomerat als vorteilhaft erscheinen ließ: Wenn die Bakterien aus einer einzigen Kolonie einer Reinkultur entnommen werden, so enthält das zu implantierende Agglomerat eine Unzahl von Einzelzellen, die bezüglich ihrer stofflichen Beschaffenheit und ihrer augenblicklichen Stoffwechsellage völlig gleichartig sind. Ein solches Implantat bietet also den gleichen Vorteil wie die Implantation von Einzelzellen (z.B. KRIEGEL, 1956). Im Gegensatz dazu stellt ein tierisches Organ einen Komplex von verschiedenen Geweben und Zellen dar, welche bezüglich der erwähnten Eigenschaften sehr verschieden sein können.

MATERIAL UND METHODE

Alle Experimente (Implantationsmethode) wurden mit Keimen von *Triturus vulgaris* im frühen Gastrulastadium (HARRISON-Stad. 10, höchstens 11 a) durchgeführt. Die Explantat (sandwich-) methode erwies sich hier als undurchführbar, da die Bakterienagglomerate ausnahmslos aus den Ektodermhüllen ausgestoßen wurden, ehe diese vollständig miteinander verwachsen waren. (Derartige Erscheinungen sind auch bei anderen Implantaten bekannt, z.B. bei *Triturus*-Leber; VAHS, 1957). Die wenigen Keime (Gastrulae), bei denen die Operationswunde nach einer Stunde noch nicht völlig verheilt war, wurden verworfen. So war die Gewähr dafür gegeben, daß der Induktor in jedem Fall im Keim verblieb. Bei der späteren histologischen Auswertung erwies sich diese Maßnahme als sehr nützlich, da die Bakterienimplantate in den meisten Fällen nicht mehr aufgefunden werden konnten.

Als Induktoren dienten Agglomerate folgender Bakterienarten: 1. Gram-positive: *Staphylococcus aureus* (SG 511), *Thermobacterium lactis* und *Corynebacterium pseudodiphtheriticum*. 2. Gram-negative: *Escherichia coli, Proteus vulgaris* und *Chromobacterium prodigiosum*.*

Implantierbare Agglomerate wurden hiervon nach folgender Methode gewonnen: Die Bakterien wurden auf geeigneten Nährböden, meistens Blut-Agar, ausgestrichen und bei 37° C so lange bebrütet, bis deutlich sichtbare Kolonien gewachsen waren. Diese wurden darauf unter dem Binokular bei 30-facher Vergrößerung mit einem ballförmigen Irismesser angehäuft und aufgenommen. So konnte mit Sicherheit vermieden werden, daß Spuren des Nährbodens mit in die Bakterienhaufen hineingeraten (Induktionsmöglichkeit!). Ältere Kulturen wurden nicht verwandt, weil gegen Ende der logarithmischen Phase und später der Gehalt an Eiweißen und Nucleinsäuren bereits beträchtlich vermindert ist. Die Bakterienklumpen kamen zur Abtötung und Koagulation eine Stunde lang in jeweils 20 ml Äthylalkohol (85%). Nun wurde tropfenweise sterile LEHMANN-Lösug (s.w.u.) hinzugegeben und gleichzeitig jeweils die gleiche Menge der Mischung abpipettiert, so daß das Volumen von 20 ml immer konstant blieb. Auf diese Weise gelangten die Bakterienklumpen nach etwa einer halben Stunde in reine, sterile LEHMANN-Lösung, in welcher sie eine weitere halbe Stunde ausgewaschen wurden. (Erfolgt die Wässerung zu plötzlich oder in zu großen Konzentrationssprüngen, so steigen die Klumpen an die Oberfläche, wo sie sofort zu einem hauchdünnen Film auseinanderfließen). Die Agglomerate, die eine weiche, leicht spröde und völlig homogene Masse darstellen, wurden nun in kleinere Brocken zerteilt, welche zu den operationsbereiten Keimen in die Operationsschälchen überpipettiert wurden. Hier lassen sie sich leicht mit der Glasnadel in implantierbare Stückchen zerlegen.

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Die Aufzucht der operierten Keime erfolgte in der von LEHMANN (1945) empfohlenen Salzlösung auf Agar-Unterlage. (In Original- und verschieden stark verdünnter HOLTFRETER-Lösung trat stets totale Exogastrulation ein). Nach etwa 14 Tagen wurden die freischwimmenden Larven protokolliert, fixiert und histologisch verarbeitet (10 μ-Paraffinschnitte; saures Hämatoxylin nach EHRLICH, essigsaures Kernechtrot).

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Experimente

1. Die Induktionsleistungen alkoholfixierter Bakterienagglomerate

Alle erwähnten Bakterienarten wurden in je einer Operationsserie (mit jeweils etwa 50 operierten Keimen) als Induktoren verwandt. Bezüglich Quantität und Qualität der Induktionsleistungen blieben die Ergebnisse durchweg weit hinter den Erwartungen zurück. In verschiedenen Serien waren überhaupt keine Anzeichen von irgendwelchen Induktionseffekten zu erkennen, weder bei der äußeren Analyse noch bei der histologischen Untersuchung. Dagegen traten in anderen Serien regelmäßig und in relativ hohen Prozentsätzen Induktionsgebilde auf, die jedoch in Masse und Umfang keineswegs mit denjenigen konkurrieren können, welche normalerweise durch tierische Gewebe hervorgerufen werden. Die Art der Verteilung dieser Induktionsgebilde auf die einzelnen Serien ließ jedoch eine bestimmte Gesetzmäßigkeit erkennen, welche den Anreiz zur Ansetzung weiterer, ursprünglich nicht geplanter Serien gab und die Problemstellung damit wesentlich erweiterte: Sämtliche gram-negativen Bakterien hatten sich als völlig unfähig zu irgendwelchen Induktionsleistungen erwiesen. Dagegen zeigten die gram-positiven regelmässige und innerhalb der drei Serien ziemlich gleichartige Induktionsleistungen. Eine getrennte Besprechung dieser drei Serien erübrigt sich daher. Die Ergebnisse sämtlicher Serien sind in Tab. 1 enthalten.

Tabelle 1. Induktionsleistungen alkoholfixierter Bakterienagglomerate

Bakterien-Arten	n	positive Fälle		Haftfäden		Linsen/ Lentoide		Epidermis-verdickgn.	
gram-positive			%		%		%		%
Staphylococcus	45	32	71	30	94	3/1	12	8	25
Thermobacterium	43	28	65	25	89	4/2	21	12	43
Corynebacterium	37	24	65	24	100	—	—	6	25
gram-negative									
Escherichia	37	—	—	—	—	—	—	—	—
Proteus	40	—	—	—	—	—	—	—	—
Chromobacterium	38	—	—	—	—	—	—	—	—

n = Anzahl der gültigen Fälle (s. Text). Die übrigen Zahlen bedeuten Fälle (Tiere), nicht Anzahl der Induktionsgebilde.

Unter der n-Zahl werden alle gültigen Fälle verstanden, d.h. solche mit positivem Induktionseffekt und solche ohne, bei denen jedoch das Implantat Kontakt mit dem Ektoderm hat. In den meisten Fällen war allerdings das Implantat nicht mehr aufzufinden, da die Bakterienhaufen offenbar schnell zerfallen und phagocytiert werden (Fig. 1, 6). Da aber einerseits die äußerst weichen und geschmeidigen Implantate nicht so leicht in den Dotter geraten und dadurch abgeschirmt werden, wie dies zuweilen bei Gewebestückchen der Fall ist, und andererseits dank der besonderen Vorsichtsmaßnahmen (s.S. 202) kaum die Möglichkeit einer unbemerkten Ausstoßung der Implantate aus der Operationswunde besteht, dürften die angegebenen n-Zahlen und folglich auch die daraus berechneten Induktionsprozentsätze weitgehend reell sein. Die allgemeinen Induktionsraten betragen demnach 71 bzw. 65%.

Die häufigsten Induktionsgebilde sind Haftfäden, von denen nicht selten mehrere (2–3), gelegentlich sogar ganze Büschel von 5–8 oder gar 11 induziert wurden (Fig. 2, 3). In einigen Fällen traten neben

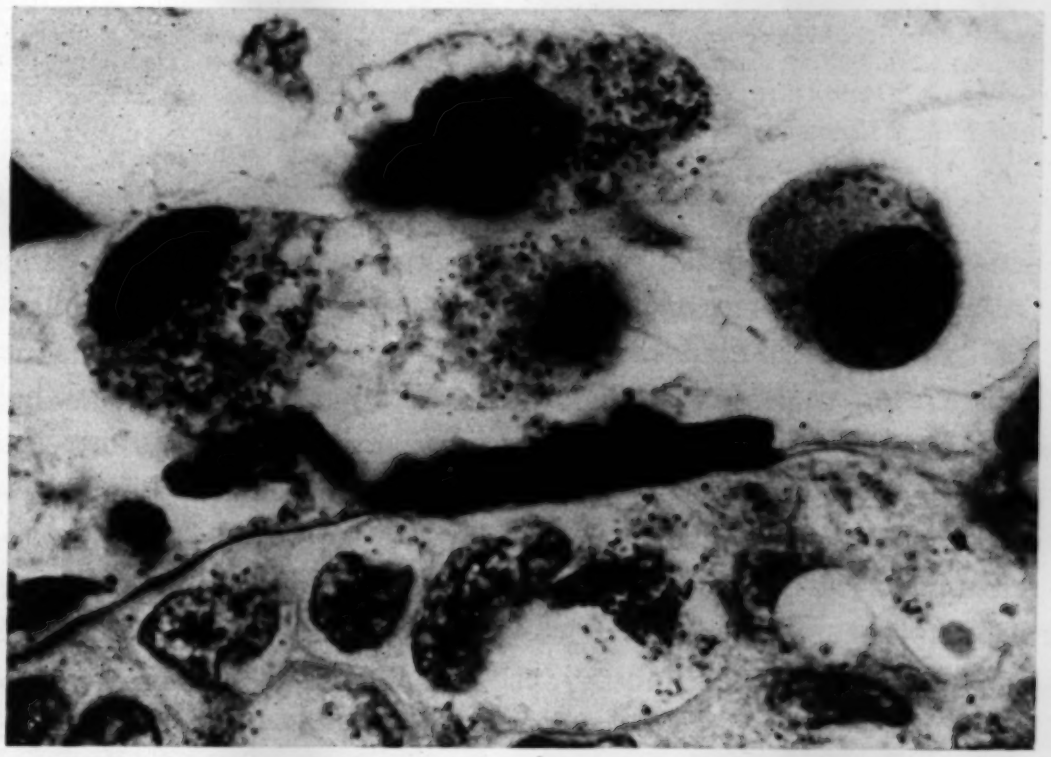


Fig. 1. Eine Gruppe von vier Phagocyten mit aufgenommenen Bakterien (*Staphylococcus aureus*). Auch das Wirtsgewebe (unten) enthält Bakterien. 1170×.

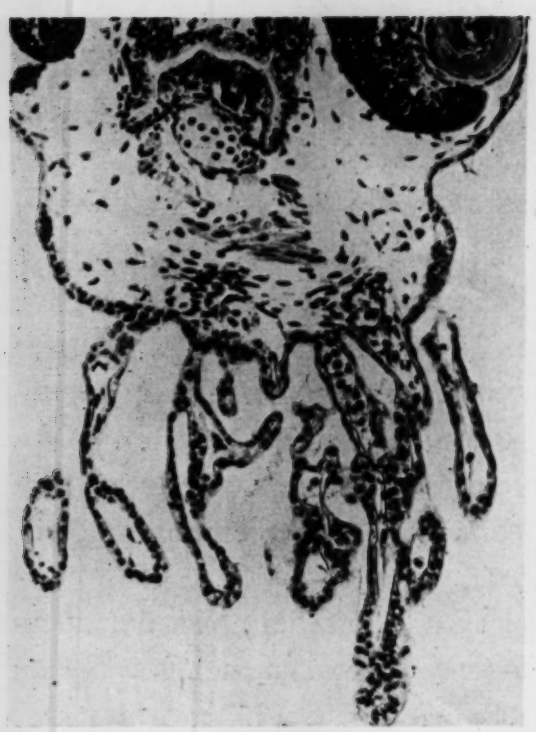


Fig. 2



Fig. 3

Fig. 2. Induktion eines Büschels von 11 Haftfäden in der Augenregion durch *Staphylococcus aureus*-Implantat. 78×.

Fig. 3. Induktion von drei miteinander verwachsenen Haftfäden und einer freien Linse in der Herzregion durch *Staphylococcus aureus*-Implantat. 155×.

diesen oder in Verbindung mit einer Epidermisverdickung (jedoch nie allein) freie Linsen oder Lentoide auf (Fig. 3, 4). Epidermisverdickungen („neuroid-epidermale" Induktionsgebilde nach HOLTFRETER, 1935 b) traten meistens allein auf. Sie nehmen, wie auch die induzierten Organe, stets eine eng begrenzte Stelle an der Ventralseite der Larve ein (Fig. 4–6). Erfahrungsgemäß deuten sie auf einen äußerst schwachen Induktionsreiz hin, wie auch die Haftfäden und freien Linsen zu denjenigen Induktionsgebilden zu rechnen sind, die ihre Hauptverbreitung in Serien mit schwachem Induktionseffekt haben. Die erhaltenen Induktionsgebilde müssen durchweg als primär induziert angesehen werden, da niemals irgendwelche anderen Organe, nicht einmal kleinste Komplexe von neuroiden Zellen, auftraten, die sie in der Eigenschaft eines sekundären Induktors hervorgerufen haben könnten. Die Haftfäden traten niemals zusammen

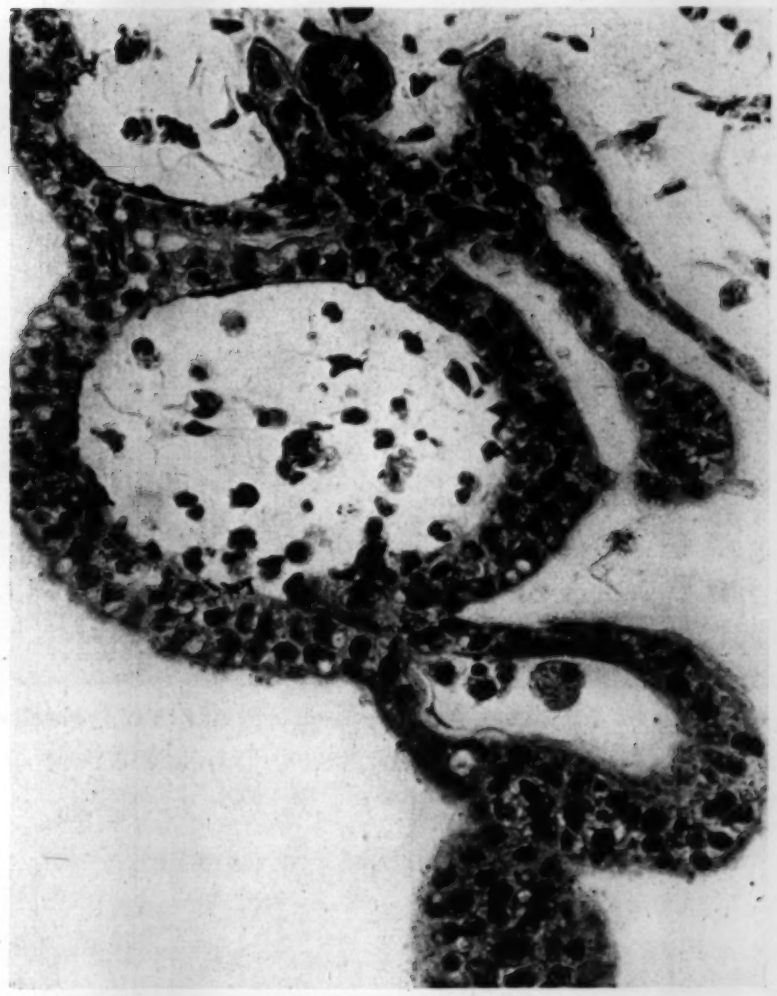


Fig. 4. Induktion von zwei Lentoiden (1 getroffen, oben), vier Haftfäden (1 paramedian getroffen, oben rechts) und einer mächtigen, blasigen Epidermiswucherung in der Herzregion durch *Corynebacterium*-Implantat. Die Blasen sind mit bakterienbeladenen Phagocyten angefüllt. 210×.

mit gleichzeitig induziertem Mesenchymgewebe auf, welches normalerweise leicht sekundär Haftfäden hervorruft.

Zusammenfassend kann also festgestellt werden, daß die grampositiven Bakterien eine deutliche archencephale Induktionsleistung entfalten, die jedoch in qualitativer Hinsicht recht schwach ist. Die freien Linsen und Lentoide erscheinen ausschließlich in der Kopf- und Herzregion, während die Haftfäden in annähernd gleichmäßiger Verteilung bis in die vordere Rumpfregion hineinreichen. Diese Art der Verteilung auf die Körperregionen des Wirtes stimmt mit den Befunden früherer Untersuchungen über den Einfluß der Wirtsregion auf das Ergebnis der ventralen Sekundärinduktion überein (Vahs, 1956).

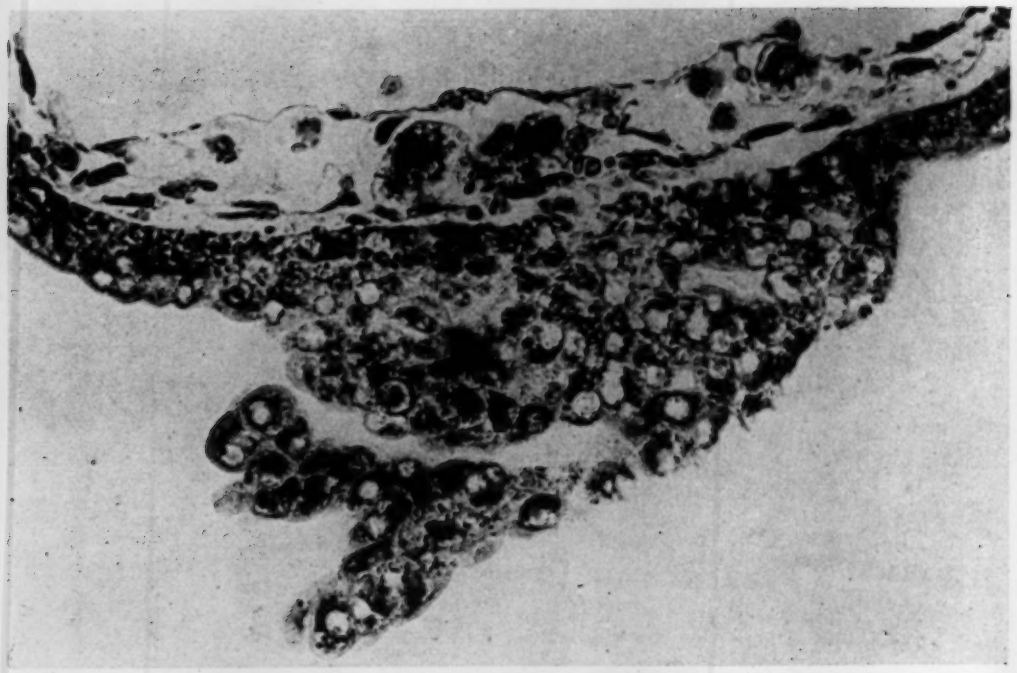


Fig. 5. Mächtige Epidermiswucherung an der Ventralseite der vorderen Rumpfregion. Innen, in der Mitte, Implantatreste (*Corynebacterium pseudodiphtheriticum*. 270×.

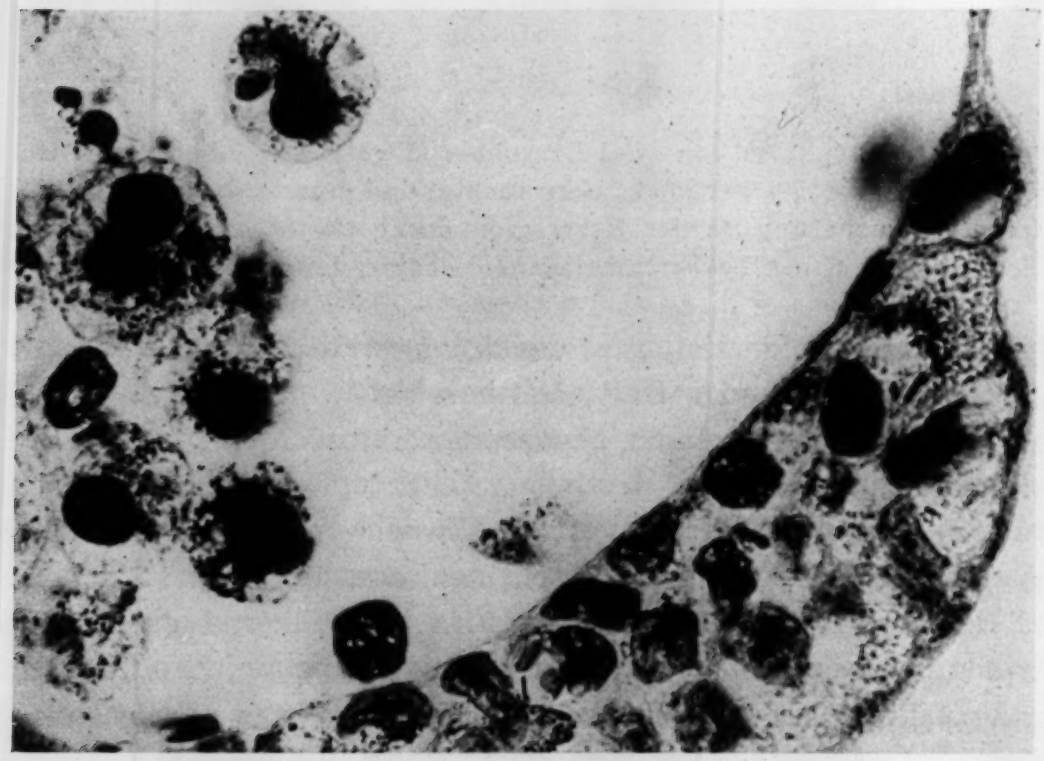
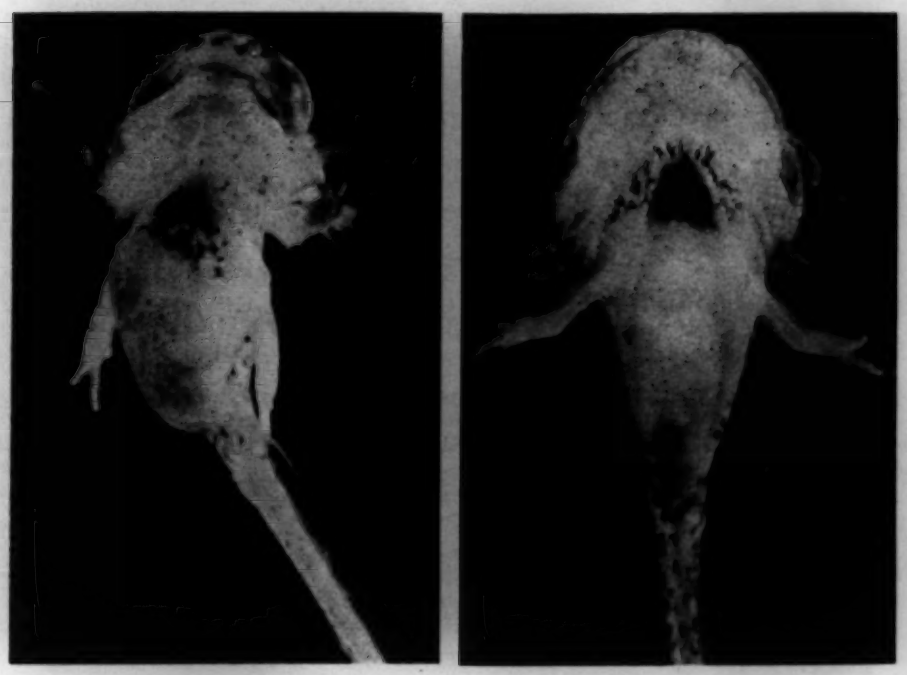


Fig. 6. Mächtige Epidermiswucherung mit Übergang zur normalen Epidermis (oben rechts). Im Inneren liegende Phagocyten (links) mit Bakterien angefüllt (*Staphylococcus aureus*). 750×.



a *b*

Fig. 7. Zwei operierte Larven im 3-Zehen-Stadium mit vervielfachten normalen Haftfäden. *a*: rechts 1, links 3; *b*: rechts 2, links 4.

Als ein recht eigenartiges Nebenergebnis sei erwähnt, daß die normalen Haftfäden (am Kopf der Larven) in allen Serien mit Haftfaden-Induktionseffekt in den meisten Fällen vervielfacht waren (meist verdoppelt, nicht selten auch büschelweise; Fig. 7). Dies war in den Serien ohne Induktionseffekt nie der Fall. Es scheint demnach, daß das Implantat in irgendeiner Weise auch die Entstehung der normalen Haftfäden begünstigt. Außerdem besteht aber auch die Möglichkeit (nach BALINSKIJ, 1937), daß das Implantat in irgendeiner Weise einen Faktor hemmt, der in der Normalentwicklung die Ausbildung von mehr als zwei Haftfäden verhindert.

Um schließlich noch die Frage zu klären, ob die relativ geringe Induktionsfähigkeit der gram-positiven Bakterien (besonders in qualitativer Hinsicht) nicht auch darauf beruhen könnte, daß die Agglomerate infolge ihres schnellen Zerfalls etwa nicht lange oder innig genug Kontakt mit dem reaktionsbereiten Ektoderm bekommen, wurden mehrere operierte Keime gegen Ende der Gastrulation (etwa HARRISON-Stad. 13) in der animalen Hälfte geöffnet. In 8 von 12 Fällen (=67%) wurde das Implantat in der ursprünglichen Beschaffenheit und in guter Kontaktsituation wiedergefunden. Die Auflösung der Implantate erfolgt also meistens in späteren Stadien,

jedenfalls erst nach Beendigung der Gastrulation. Demnach muß die geringe Induktionsfähigkeit mit den in den Bakterien enthaltenen Stoffen in direktem Zusammenhang stehen. Die negativen Fälle dürften dagegen auf eine ungenügende oder unterbliebene Kontakterhaltung zurückzuführen sein, wie aus der guten Übereinstimmung der Prozentsätze der wiedergefundenen Implantate (67%) mit denen der allgemeinen Induktionsraten (71, 65, 65%; Tab. 1) hervorzugehen scheint. So kann man auf Grund der vorstehenden Befunde annehmen, daß die wirklichen Induktionsraten der gram-positiven Bakterien im Idealfall, d.h., wenn immer guter Kontakt erreicht wird, nahezu 100% betragen.

2. Prüfung künstlich gram-negativ gemachter Bakterien auf Induktionsfähigkeit

Da die gram-negativen Bakterien im Gegensatz zu den gram-positiven keine Induktionswirkung gezeigt hatten, lag es nahe, zu prüfen, wie sich diesbezüglich die letzteren verhalten, wenn sie künstlich gram-negativ gemacht werden. Dies läßt sich durch Eliminierung des Gram-Stoffes (s. Diskussion) erreichen, und zwar entweder durch Herauslösung desselben nach der Alkoholfixation oder durch seine Zerstörung in den lebenden Bakterien vor der Fixation. Von diesen Möglichkeiten wurden vier besonders geeignete ausgewählt und teils bei allen drei Bakterienarten, teils bei je zwei Arten angewandt (Tab. 2).

Tabelle 2. Anwendung der verschiedenen Methoden zur Eliminierung des Gram-Stoffes bei den einzelnen Bakterienarten (n-Zahlen)

gram-pos. Bakterien / behandelt mit:	Cholat	RN'ase	*n*-HCl	UV
Staphylococcus aureus	24	20	22	21
Thermobact. lactis	22	—	25	—
Corynebact. pseudod.	23	19	—	18

a. Behandlung mit gallensauren Salzen (Cholaten)

Nach Fixierung und Auswaschung wurden die Bakterienagglomerate in eine 2%ige Gallensalzlösung pipettiert, welche 2/3 Na-Cholat und 1/3 Na-Taurocholat (SCHUCHARDT, München) enthielt. Die Inkubation erfolgte 12 Std. bei 60° C unter ständiger, sehr feinblasiger Durchströmung mit reinem Sauerstoff.

b. Behandlung mit Ribonuclease

Die (bei 0° C aufbewahrte) Ferment-Stammlösung enthielt 2 mg kristallisierte Ribonuclease (SCHUCHARDT, München) pro ml N/100 HCl, so daß nach Einstellung auf pH 7,0 mit N/100 NaOH eine Konzentration von 1 mg Ferment pro ml Lösung erhalten wurde. Der dabei auftretende Gehalt an NaCl ist so gering (0,029%), daß er nicht extrahierend wirkt. Mit dieser Fermentlösung wurden die fixierten und ausgewaschenen Bakterienagglomerate 4 Std. bei 37° C behandelt.

c. Behandlung mit N-HCl

Nach Fixierung und Auswaschung erfolgte die Inkubation 4 Std. bei 37° C. Anschließend wurden die Agglomerate, wie auch bei den Behandlungen nach *a* und *b*, gründlich in steriler LEHMANN-Lösung ausgewaschen, bevor sie implantiert wurden.

d. Bestrahlung mit bakteriziden Ultraviolett-Dosen

Die Bakterienkulturen wurden mit einem Quarzbrenner der Marke „Original Hanau", Typ Q 250, in einem Abstand von 45 cm senkrecht bestrahlt. Die UV-Bestrahlungsstärke betrug hierbei für 200–400 mμ 6 mW/cm², für 240–280 mμ (= UV-c) 2,5 mW/cm². In einem Vorversuch wurde festgestellt, daß frisch ausgestrichene Bakterien nach 20 Minuten langer Bestrahlung keine Spur von Wachstum mehr zeigen (Nachschau 48 Std. nach der Bestrahlung). Nun wurden frisch angesetzte und etwa 10 Std. lang bebrütete Kulturen unter denselben Bedingungen je eine Stunde lang bestrahlt, nach weiteren 12 Std. alkoholfixiert und, wie oben beschrieben, zu Implantaten verarbeitet.

e. Kontrollversuche zu den Methoden a—d

Vor und nach den verschiedenen chemischen Behandlungsweisen (*a-c*) wurden Proben der Agglomerate zu Ausstrichpräparaten verarbeitet und einer standardisierten Gram-Färbung unterworfen. In allen Fällen waren die Bakterien vor der Inkubation stark grampositiv, nachher eindeutig und 100%ig gram-negativ. Dasselbe gilt für die Bakterien unmittelbar nach der Bestrahlung bzw. 12 Std. darauf.

Ferner wurde die Wirkung der chemischen Agenzien auf folgende Weise geprüft: Histologische Schnitte von Mäuseleber

zeigten ohne die betreffende chemische Behandlung nach Färbung mit Methylgrün-Pyronin oder Toluidinblau starke cytoplasmatische Basophilie, welche bei behandelten Präparaten (Ausführung unter denselben Bedingungen wie unter *a–c*) vollständig vernichtet war.

f. Ergebnisse der Implantationsversuche mit den nach a—d behandelten Bakterienagglomeraten

In allen Fällen erwiesen sich die behandelten Bakterienagglomerate als völlig induktionsunfähig. Nicht einmal Epidermisverdickungen (als Anzeichen von schwächsten Induktionseffekten) waren zu verzeichnen. Auch die sonst häufig beobachtete Vervielfachung der normalen Haftfäden (s.S. 209) trat nie auf. Demnach gleichen also die künstlich gram-negativ gemachten Bakterien bezüglich ihrer Induktionsunfähigkeit den von Natur aus gram-negativen Arten.

Da die chemische Natur desjenigen Stoffes, welcher die Grampositivität bedingt, in letzter Zeit ziemlich sicher aufgeklärt werden konnte und auch die Wirkungsweisen der verwendeten chemischen und physikalischen Manipulationen weitgehend bekannt sind, geben die verzeichneten Ergebnisse aufschlußreiche Hinweise darauf, welche Stoffe unter den gegebenen Versuchsbedingungen an der Entstehung der Induktionsgebilde beteiligt sein müssen.

Diskussion

Die Ergebnisse der vorliegenden Untersuchungen beweisen, daß auch Bakterien (in geeigneter Aufbereitung) Induktionsgebilde hervorzurufen vermögen. Jedoch kommt diese Fähigkeit nur den grampositiven Arten zu, welche als die höheren Formen des Bakterienreiches angesehen werden. Da wir sowohl unter den gram-positiven als auch unter den gram-negativen Bakterien absichtlich mehrere Vertreter aus den verschiedensten systematischen Gruppen ausgewählt haben, darf man diese Feststellung wohl verallgemeinern. Bei relativ hoher Induktionsrate sind die Leistungen jedoch nur mit denjenigen schwach wirksamer tierischer Gewebe zu vergleichen. Andererseits übertreffen sie bezüglich ihrer Induktionsleistungen die Implantate pflanzlichen Ursprungs erheblich. Als echte Induktionsgebilde wurden vorwiegend Haftfäden, gelegentlich auch freie Linsen und Lentoide festgestellt. Der Charakter der Induktion ist also archencephal. Daß die fast ausschließliche Induktion von Haftfäden

der Ausdruck einer äußerst schwachen Induktionsfähigkeit (in qualitativer Hinsicht) ist, geht auch aus anderen Untersuchungen hervor: So vermag z.B. die normalerweise sehr stark induzierende Mäuseniere nach besonders drastischen Eingriffen in das Stoffgefüge (trockene Erhitzung bei 100° C) nur noch Haftfäden zu induzieren, jedoch in hohen Prozentsätzen (VAHS, 1955).

Der schroffe Gegensatz zwischen gram-positiven und -negativen Bakterien bezüglich ihrer Induktionsfähigkeit macht es sehr wahrscheinlich, daß die induzierende Wirkung auf den Gram-Stoff zurückzuführen ist. Die chemische Natur dieser nur in gram-positiven Mikroorganismen vorkommenden Substanz konnte durch HENRY und STACEY (1943), sowie HENRY, STACEY und TEECE (1945) aufgeklärt werden: Gallensalzextrakte gram-positiver Mikroorganismen ergaben eine Alkoholfällung, die als Mg-Salz eines Ribonucleoproteids identifiziert werden konnte. Das übrigbleibende Cytoskelett erwies sich nach der Extraktion als gram-negativ. Die Nucleinsäure- und die Proteinkomponente waren je für sich gram-negativ; aber das wiederhergestellte Nucleoproteid, das sich in Gegenwart von Formaldehyd (Reduktion) und Magnesium wieder in das gram-negative Bakterienskelett einfügen läßt, gibt diesem die Gram-Positivität zurück.

FOSTER und WILSON (1951) fanden, daß Lösungen gallensaurer Salze die (durch RNS verursachte) cytoplasmatische Basophilie von Gewebsschnitten (HVL) vernichten. Weiterhin berichtet STACEY (1955), daß gram-positive Bakterien auch durch Ribonuclease gram-negativ gemacht werden können. Demnach sind die Wirkungen beider Agenzien weitgehend ähnlich: Sie extrahieren spezifisch RNS-Komplexe. In den besprochenen Induktionsexperimenten mit gram-positiven Bakterien vernichteten sie deren Induktionsfähigkeit.

Auch nach Extraktion der Bakterien mit N-HCl, welche neben RNS auch DNS entfernt (Hydrolyse), war jede Induktionsfähigkeit erloschen.

Ultraviolett-Strahlung vermag bekanntlich Bakterien und andere Mikroorganismen abzutöten, besonders im kurzwelligen Gebiet des UV-Spektrums (UV-c; $\lambda < 280$ mμ). Die erforderliche Strahlungsdosis wurde bei den geschilderten Experimenten erreicht, wie die Kultur- und Färbeversuche gezeigt haben. Die zelltötende Wirkung der

UV-Strahlen ist allgemein verbreitet und stellt bei den Bakterien als einzellige Organismen nur einen Spezialfall dar.

Die Wirkung besteht einmal in einer Entfaltung der Eiweiß-Makromoleküle und Sprengung der Peptidbindungen (= Denaturierung). Auf eine irreversible Oxydation der Proteine und Aminosäuren folgt schließlich eine allgemeine Proteolyse (GIESE, 1950; FRASER, 1952). Die Proteinsynthese wird unterbunden, weil andererseits auch die (hierzu erforderlichen) Nucleinsäuren zerstört werden, welche offenbar die eigentlichen „Rezeptoren" der Zelle für UV darstellen (ERRERA, 1953). MITTWER (1953) berichtet, daß der Gram-Stoff in Bakterien und Hefen durch UV-Strahlen vernichtet wird. Da diese Vorgänge in ihrer Gesamtheit eine gewisse Zeit beanspruchen, haben wir die bestrahlten Bakterien so lange aufbewahrt, bis sie 100%ig gram-negativ geworden waren (*ca.* 12 Std.) und erst dann zu Implantaten verarbeitet. Sie erwiesen sich, wie auch nach den chemischen Behandlungen, als völlig induktionsunfähig.

Die Ergebnisse der besprochenen Experimente zeigen also, daß die Entstehung der erhaltenen Induktionsgebilde (Haftfäden und freie Linsen) unter den vorliegenden Versuchsbedingungen abhängig war vom Vorhandensein eines Ribonucleoproteids (Gram-Stoff). Bei früheren Untersuchungen mit tierischen Geweben als Induktoren haben wir die Vermutung ausgesprochen, daß Eiweißkörper vom Nucleoproteidtypus für die spinocaudale Induktion zuständig sind (VAHS, 1957). Umso bemerkenswerter ist es, daß solche im vorliegenden Fall auch bzw. nur archencephale Strukturen, wenn auch nur sehr „schwache", hervorbrachten.

Aus den vorliegenden Ergebnissen kann jedoch nicht geschlossen werden, daß die archencephale Induktion schlechthin durch Ribonucleoproteide verursacht werde, da echte archencephale Gebilde, wie Teile oder Derivate des Archencephalons (Hirnteile, Augen, Nasen), nicht auftraten. Haftfäden und Linsen sind in der Normogenese sekundäre Induktionsgebilde und werden nur deshalb zu den archencephalen Gebilden gerechnet, weil sie im Experiment nur zusammen mit echten archencephalen Organen bzw. nur in Serien mit archencephalem Induktionseffekt auftreten. Die Bezeichnung „archencephal" hat hier also rein klassifizierenden Wert, ist aber allgemein üblich (vgl. hierzu YAMADA und TAKATA, 1956). Was speziell die

Haftfäden anlangt, so sei darauf hingewiesen, daß diese offenbar durch verschiedene Induktionseinflüsse hervorgebracht werden können, wie wir bereits an anderer Stelle ausführlich diskutiert haben (VAHS, 1956).

Nach den neueren Untersuchungen von HAYASHI (1956), YAMADA und TAKATA (1956) scheint es aber doch, daß nicht nur die spinocaudal-deuterencephale, sondern auch die archencephale Induktion auf Ribonucleoproteide zurückzuführen ist: Ribonucleoproteide, welche aus Meerschweinchenorganen extrahiert worden waren, zeigten nämlich die ursprüngliche Induktionsleistung des intakten Gewebes: spinocaudal-deuterencephale (Niere bzw. Extrakt) und archencephale (Leber bzw. Extrakt). Bezüglich desjenigen Teiles der archencephalen Induktion, welcher bei den Bakterienexperimenten erfaßt werden konnte, stehen demnach unsere eigenen Ergebnisse in gutem Einklang mit denen der japanischen Forscher.

Schließlich ist noch die Frage zu behandeln, warum die Bakterien trotz ihres außerordentlich hohen Gehaltes an Nucleinstoffen und Eiweißen das Gastrulaektoderm nur zur Ausbildung der schwächsten Induktionsgebilde veranlassen können. Ganz allgemein bleibt zu klären, worin die erheblichen Unterschiede zwischen den Induktionsleistungen tierischer und nichttierischer Gewebe bzw. Zellen begründet sein könnten. WOERDEMAN (1933) und HOLTFRETER (1935 a) konnten zeigen, daß z.B. Glykogen, das bei höheren Pflanzen fehlt und daher ursprünglich als möglicher Induktionsfaktor der tierischen Gewebe angesehen wurde, nicht als solcher in Frage kommt. Das ist auch aus den vorliegenden Untersuchungen zu schließen, denn Glykogen stellt das verbreitetste Reservekohlenhydrat der Bakterien dar. Bezüglich der Proteine und Nucleinstoffe, welche ja in sämtlichen Organismen vorkommen, können die erwähnten Leistungsunterschiede nicht auf quantitativen, sondern nur auf qualitativen Unterschieden beruhen. Sonst hätten z.B. die Bakterien besonders starke Induktionswirkungen zeigen müssen. Außerdem wäre sonst nicht zu verstehen, warum die Pflanzen so gut wie keine Induktionsfähigkeit besitzen.

Die neueren Erkenntnisse der Chemie der Makromolekularen können diesen Fragenkomplex einer Klärung näherbringen. Unter den Makromolekularen sind die Eiweißkörper und die Nucleinsäuren

besonders wichtige Stoffe, da sie in engster Verbindung miteinander die wesentlichste Eigenschaft der lebenden Zelle, ihre Selbstreproduzierbarkeit, bewirken. Sehr wahrscheinlich besitzt jede Organismenart für sie eigentümliche, spezifische Eiweiße (vgl. die Reizwirkung „artfremder" Eiweiße). Dabei unterscheiden sich sogar die Eiweiße verschiedener Organe desselben Organismus. Derartige Verschiedenheiten können einmal auf Unterschieden in Art, Zahl und Reihenfolge der Aminosäurebausteine innerhalb der Peptidketten beruhen, zum anderen (bei gleicher chemischer Zusammensetzung) auf der Gestaltspezifität, die sich in verschiedenartiger Faltung und Anordnung der Peptidketten innerhalb der Makromoleküle äußert. Die ungeheuer große Zahl (200–300 Stellen) der unter diesen Aspekten möglichen Strukturvariationen wird zwar in der Natur nicht erreicht; dennoch ist aber eine sehr große Zahl verschiedener Eiweißkörper bekannt, deren Spezifität offenbar mit ihrer biologischen Funktion zusammenhängt. Auch ist bekannt, daß die Eiweißkörper von Tieren und Pflanzen sich in mannigfacher Hinsicht unterscheiden.

Bei den Nucleinstoffen bestehen ganz ähnliche Verhältnisse. Sie erreichen bezüglich ihrer stofflichen Zusammensetzung und Struktur ebenfalls hohe Grade der Mannigfaltigkeit, zumal ihre Monomeren nicht, wie bei den Proteinen, zu derselben Stoffklasse (Aminosäuren), sondern zu verschiedenen gehören (Phosphorsäure, Kohlenhydrate, verschiedene Stickstoffbasen). Bei verschiedenen Nucleoproteiden kann weiterhin das Verhältnis der Nuclein- zur Proteinkomponente und auch die Art ihrer Bindung unterschiedlich sein. Ribonucleinsäuren aus verschiedenen Tieren sind einander ähnlicher als tierische, verglichen mit pflanzlichen.

Der weitaus größte Teil des Bakterieneiweißes ist an Nucleinsäuren gebunden, sofern es sich nicht um Enzymeiweiß handelt. So ist das eigentliche Reserveeiweiß der Bakterien, das sog. Volutin, ein Nucleoproteid, welches die „Volutin-Nucleinsäure" enthält, eine RNS mit esterartig gebundener Schwefelsäure. Diese Substanz ist sonst nur noch bei Algen, Pilzen und Protozoen (?) bekannt. Weiterhin unterscheiden sich die Bakterien-Nucleoproteide von denjenigen der höheren Organismen durch ihre sehr schwere Löslichkeit in Salzlösungen. Unterschiede zeigen sich auch darin, daß die bei tierischen Geweben üblichen Methoden der Isolierung bei Bakte-

rien fast stets versagen. Alle bisher untersuchten Bakterienarten erforderten diesbezüglich ganz spezielle, „individuelle" Methoden (CHARGAFF, 1947). Ferner darf nicht übersehen werden, daß ja auch die gram-negativen Bakterien reich an Nucleinsäuren und Eiweißen sind. Diese besitzen aber im Gegensatz zu denjenigen der gram-positiven keine Induktionsfähigkeit, was auf stoffliche oder strukturelle Unterschiede irgendwelcher Art schließen läßt.

Trotz der erwähnten, theoretisch unfaßbar großen Variationsmöglichkeiten im Aufbau der Makromolekularen sind aber dennoch weder die Aminosäuren in den Peptidketten noch die Mononucleotide in den Polynucleotidketten (=Nucleinsäuren) wahllos unter Ausnutzung aller variationsstatistischen Möglichkeiten angeordnet, sondern es gibt bestimmte Regelmäßigkeiten, wahrscheinlich durch periodische Wiederkehr von Rhythmen gleicher oder ähnlicher Bausteine.

So ist es denkbar (und zum Teil sehr wahrscheinlich), daß unter den zahlreichen verschiedenen Ribonucleoproteiden einzelne große Gruppen zu unterscheiden sind, deren Vertreter untereinander zwar auch gewisse Unterschiede aufweisen, sich aber dennoch in bestimmten Punkten ähnlich sind. Diejenigen der (meisten) tierischen Gewebe besitzen infolge einer bestimmten Zusammensetzung oder Struktur, d.h. dank bestimmter physiologischer Eigenschaften u.a. die Fähigkeit zu induzieren, während diese bei anderen Gruppen fehlt (pflanzliche) oder nur zu schwachen Induktionswirkungen reicht (bakterielle Nucleoproteide).

SUMMARY

(1) Since most of the animal tissues, as opposed to vegetal ones, are capable of more or less strong inductive effects, bacteria as representatives of the third kingdom of organisms have been tested for such effects, the tests having been conducted especially with regard to their extremely high content of proteins and nucleic acids (especially RNA). Pure cultures of various bacteria were agglomerated by 85% alcohol, then thoroughly washed with LEHMANN's solution and finally implanted into early gastrulae of *Triturus vulgaris*. The following species were applied: 1. gram-positive bacteria: *Staphylococcus aureus*, *Thermobacterium lactis*, and *Coryne-*

bacterium pseudodiphtheriticum. 2. gram-negative bacteria: *Escherichia coli*, *Proteus vulgaris*, and *Chromobacterium prodigiosum*.

(2) Whereas the gram-negative bacteria showed no inductive effect at all, the gram-positive regularly produced a relatively high degree of inductive formations. Among these balancers were predominant, whereas free lenses and/or lentoids, and epidermis thickenings occured only occasionally. Thus the induction shows an archencephalic character the quality of which, however, is like that of a very weakly effective animal tissue, but, on the other hand, exceeds that of the vegetal tissues.

(3) In order to eliminate or to destroy the gram-material the gram-positive bacteria were subjected to the following treatments prior to implantation: Extraction by bile-salt solutions and N-HCl respectively, treatment with ribonuclease, and irradiation with bactericidal ultraviolet doses. In each case the treated bacteria became 100% gram-negative. After that they were no more able to induce than are those which are gram-negative by nature.

(4) From these results ((2) and (3)) it is concluded, that, under the present experimental conditions, there is a close connection between the induction phenomenon and the gram-positive material, the latter being known as a Mg-ribonucleoprotein.

(5) With the aid of the recent knowledge of macro-molecular chemistry an attempt was made to explain the considerable differences in inductive power between implants of animal, vegetal, and bacterial origin.

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INFLUENCE OF SODIUM IODIDE AND SODIUM THIOCYANATE UPON THE DEVELOPMENT OF FROG'S EMBRYOS

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INTRODUCTION

According to RANZI and TAMINI (1939) and RANZI (1945) a treatment of the amphibian embryo with sodium thiocyanate caused enlargement of the neural canal and bending of the notochord. The total volume of the notochord of treated embryos was found larger than that of the control (RANZI and TAMINI, *l.c.*), and the notochord anlage of the tail-bud stage contained a larger number of cells as compared with that of the control (RANZI, *l.c.*). The morphogenetic effects of sodium thiocyanate were interpreted as supernormal development ("ipersviluppo") of the notochord, which may lead to supernormal induction of the neural system. On the contrary, the suppressive effect of lithium chloride on the differentiation of the notochord in the amphibian embryo has been well known (cf. LEHMANN, 1938). The above results consequently lead to the suggestion that the antagonism of morphogenetic effects of lithium chloride and sodium thiocyanate established in the echinoderm development can also be extended to the amphibian development.

However the present writer found only infrequent occurrence of the notochord with enlarged cross section in amphibian embryos treated with sodium thiocyanate (ÔGI, 1955). On the other hand, a very high incidence of twisted notochord was recorded in the same material. All notochords with enlarged cross section were found to be twisted. The impression was obtained that the observed enlargement of the notochordal cross section in a restricted part of the organ might be a consequence of morphogenetic disturbance but not of a supernormal development of the anlage.

The first half of the present paper deals with the development of

amphibian embryos treated with sodium iodide, which may have an effect similar to that of thiocyanate as judged from its animalizing effect on the echinoderm egg (LINDAHL, 1936), as well as from its viscosity lowering effect on protein solution (RANZI, 1952). In the second half of this paper attempts are made to estimate the cell number and cell size of the notochord malformed by the treatment with sodium thiocyanate and iodide.

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MATERIALS AND METHODS

For studying effects of sodium iodide the embryos of *Bufo vulgaris* from single batch were used exclusively. Cleavage-, morula-, blastula-, early and late gastrula-stages were used. A group of 50 individuals each with intact jelly coat were immersed in solutions of M/10, M/20 and M/30 sodium iodide for 24 hours. The embryos were washed thoroughly and kept in tap water at room temperature. The embryos treated at blastula-stage had not started gastrulation when they were returned to tap water. The culture was continued to the external gill stage at which surviving embryos were fixed in BOUIN's fluid. When embryos were found dying they were fixed immediately regardless of the stage. Embryos were either stained *in toto* with borax carmine and sectioned, or stained in section with DELAFIELD's hematoxylin. The sections were used for morphological observation as well as for cell counts.

In sodium thiocyanate-series the embryos of *Bufo vulgaris* and *Rana nigromaculata* were used as materials. The former was treated with M/30 sodium thiocyanate for 24 hours from late blastula- to gastrula-stage. The latter was treated with M/40 for 12 hours during the same period. Both of them were used for estimation of the cell number of the notochord on sections prepared as mentioned above.

RESULTS

A. External observations of the embryos treated with sodium iodide

After 24 hours' treatment although the development of the

embryos was delayed in proportion to the concentration, no special morphological abnormality could be observed. On further culture in tap water the following types of abnormalities were found to occur:

a) *Severe disturbances of invagination.* The greater part of the vegetal hemisphere remained exposed as a large yolk plug. In these embryos the neural plate was abnormally shortened, suggesting a subnormal activity of the organizer. Often the epidermis was much

Table 1. Frequency of abnormalities in iodide-treated embryos

Stage of treatment	External observation	Concentration of NaI		
		M/10	M/20	M/30
Cleavage	Dead	48	1	21
	Embryo with permanent yolk plug	0	43	9
	Double tailed embryo	0	0	2
	Kyphosis	0	0	3
	Normal	0	0	14
Morula	Dead	6	1	0
	Embryo with permanent yolk plug	45	42	42
	Double tailed embryo	0	0	0
	Kyphosis	0	2	18
	Normal	0	1	6
Blastula	Dead	24	2	1
	Embryo with permanent yolk plug	13	45	20
	Double tailed embryo	0	0	0
	Kyphosis	0	2	18
	Normal	0	1	6
Early gastrula	Dead	51	6	0
	Embryo with permanent yolk plug	0	39	29
	Double tailed embryo	0	1	0
	Kyphosis	0	5	9
	Normal	0	1	12
Late gastrula	Dead	8	1	1
	Embryo with permanent yolk plug	0	0	0
	Double tailed embryo	0	0	0
	Kyphosis	3	0	3
	Normal	6	46	45

wrinkled. However in sections the marginal zone was found invaginated under the ectoderm.

b) *Spina bifida.* The tail was bifurcated and bent toward the dorsal side. Some of the embryos were provided with a conspicuous protuberance on the hind flank.

c) *Kyphosis.* The development of the tail was retarded as in the case of thiocyanate-treatment and bent ventrally. Also the kyphosis was often accompanied by the protuberance on the hind flank.

All the treated embryos, regardless of the types of abnormalities, showed an edematous tendency.

B. Differentiation of germ layers in the embryos treated with sodium iodide

As the microscopic observation revealed that the abnormalities are generally similar to those found in the thiocyanate-series reported elsewhere (Ôgi, 1955), the following description will be limited to those which are not in common with the thiocyanate-series or to those which seem to be of general importance.

a. Ectoderm. The epidermis often assumed the wrinkled, irregular surface, as observed earlier in thiocyanate-series. Occasionally suckers and sensory organs showed hypomorphic tendency which may lead to the absence of one or both of the partners. The development of the central nervous system was more or less severely disturbed. In extreme cases the brain was represented by a mere thickening of the ectoderm or a mass of neural cells, while in other cases the brain was found with a remarkably thickened wall and an extremely reduced ventricle. Presence of a large number of free cells within the ventricle also suggested a disturbance in the neural system. The spinal cord of some embryos showed abnormal branches (Figs. 2, 3) or many irregular lumina (Fig. 4).

b. Notochord. The notochord of the severely affected embryo was composed of imperfectly vacuolized cells containing much cytoplasm. In other cases the notochord was well differentiated with perfectly vacuolized cells. However the following special deformation was often observed: (1) In the anterior part the notochord sent out a projection upwards or downwards, which penetrated the adjacent tissues,

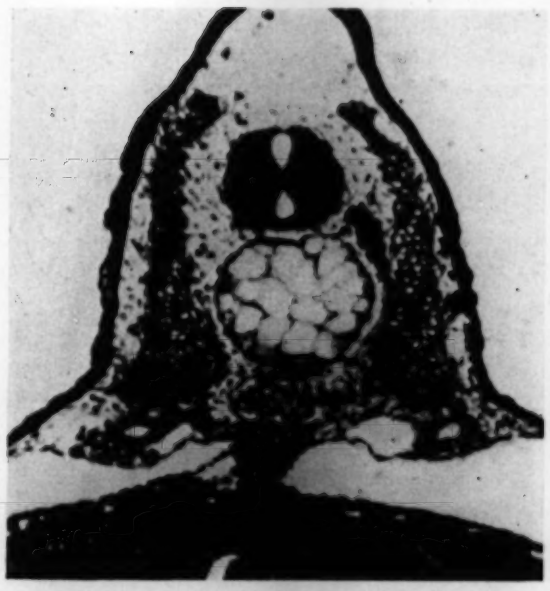


Fig. 1



Fig. 2



Fig. 3

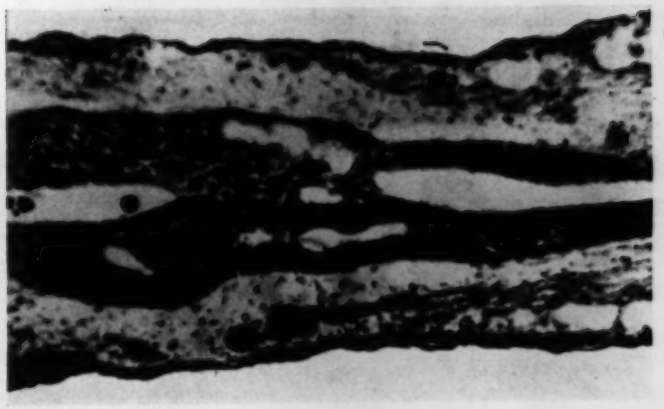


Fig. 4

Fig. 1. A cross section of the control embryo (×70).

Figs. 2, 3. Representing tow cross sections through the anterior trunk region, showing branched spinal cord and "enlarged" notochord (×70). Fig. 3, section at the level immediately caudal to that shown in Fig. 2.

Fig. 4. A frontal section showing many irregular lumina in the spinal cord (×70).

such as the brain (Fig. 6) or the dorsal wall of the foregut (Figs. 5, 7). In this latter case the projection was surrounded by a thickened layer of the endoderm (Fig. 8). (2) In the kyphosis the diameter of the notochord was about 1.5 times as large as that of the normal one (Figs. 2, 3). However this does not necessarily mean an increase in the total amount of the notochordal tissue, as will be shown in a later

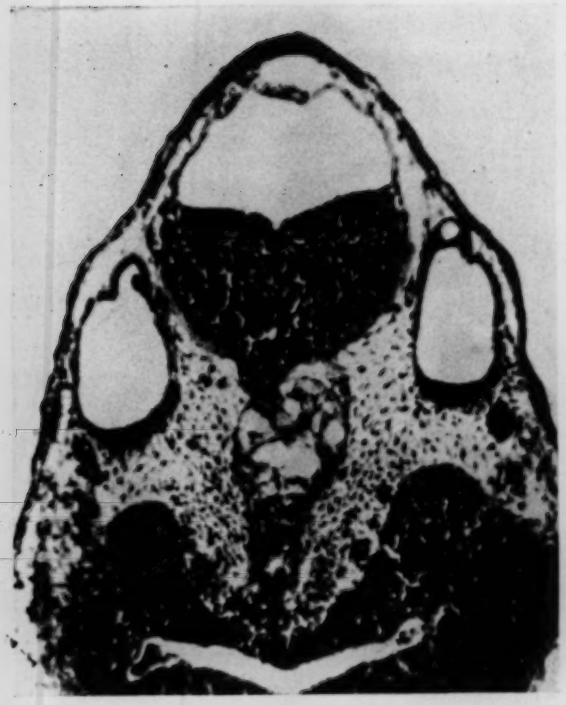


Fig. 5



Fig. 6



Fig. 7



Fig. 8

Fig. 5. Thickening of the wall of the hind brain and a ventral projection of the notochord ($\times 70$).

Fig. 6. A notochordal process in the wall of the brain ($\times 70$).

Fig. 7. A cross section showing the thickened neural tube and notochordal process toward the ventral side ($\times 70$).

Fig. 8. A cross section through the same embryo as in Fig. 7 at a more caudal level, showing the projection of notochord embedded in the enteric roof ($\times 70$).

Table 2. Frequency of notochordal abnormalities and ventral protuberance in iodide-treated embryos

Stage of treatment	Externally observed malformations	Total number	Branched notochord	Enlarged notochord	Cell mass in blood island
Cleavage	Double tailed and kyphosis (M/30)	5	0	0	0
Morula	Permanent yolk plug (M/30)	13	7	0	3
Blastula	Permanent yolk plug (M/20)	10	6	0	3
	Kyphosis (M/20)	2	0	0	1
	" (M/30)	18	5	10	10
Early gastrula	Double tailed (M/20)	1	1	0	0
	Kyphosis (M/20)	5	1	0	1
	" (M/30)	9	0	6	8
Late gastrula	Kyphosis (M/30)	3	1	0	2

section. The frequency of the branched and thickened notochord is shown in Table 2.

c. Mesoderm. The mesenchyme, myotome, nephrotome and lateral plate were well developed except in some embryos in which the differentiation of all tissues was inhibited. In many embryos which survived until later stages, a cell mass was observed in the region of blood island, sometimes forming a remarkable protuberance in the post-abdomen (Figs. 9, 10). This cell mass was found in the vitelline vein and seemed to be formed by undifferentiated mesoderm cells. Probably these cells represent the presumptive material for the blood island, which is inhibited in its development by the treatment, and has not undergone normal blood cell differentiation. The frequency of such condition is also included in Table 2.

C. The total number of cells in the notochord of iodide- and thiocyanate-treated embryos

In order to decide whether or not the notochord abnormalities obtained by the iodide- and thiocyanate-treatments are associated with an increase in total cell number of this tissue, the number of cells of the notochord in treated embryos was counted. For estimating the cell number, counting of nuclei is generally more reliable than direct cell count. However in the differentiated notochord as the nucleus

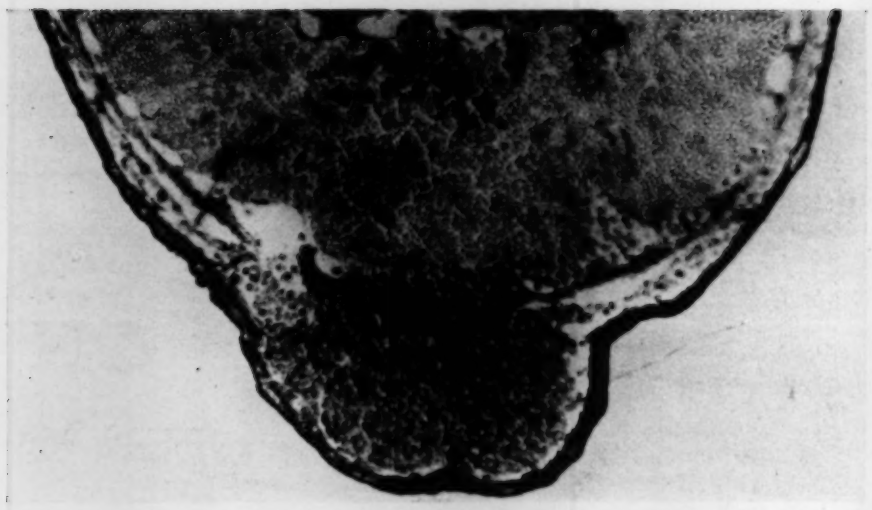


Fig. 9

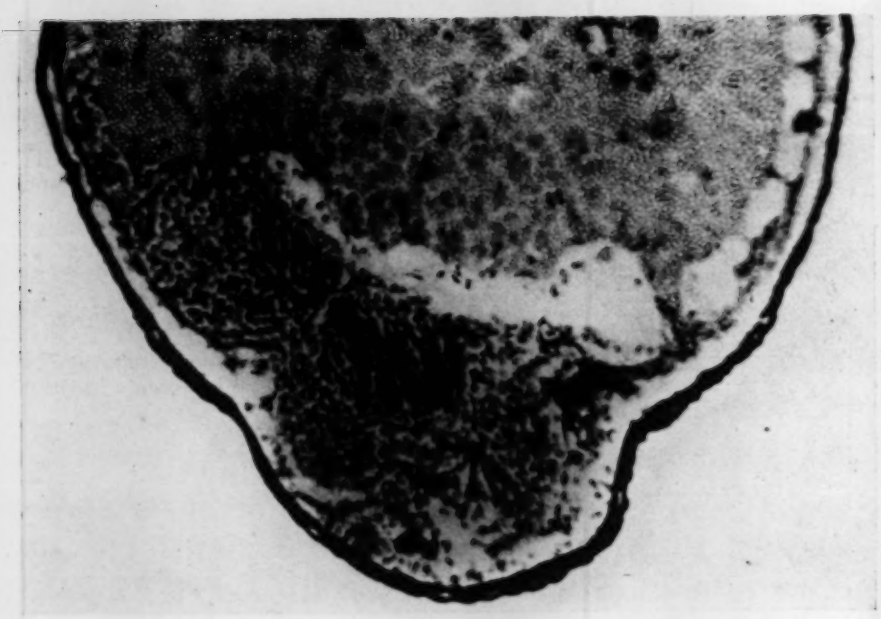


Fig. 10

Fig. 9, 10. Cross sections through the posterior region of the abdomen with the large protuberance, showing the blood cell-like mass lying in the vitelline vein (×70).

is extremely deformed by vacuolization of the cytoplasm and consequently difficult to count in section, direct count of cell number was practiced in series *a*, admitting technical drawback of the method. In series *b*, the nuclei count was tried on the early notochord rudiment before onset of intensive vacuolization.

Series a. Direct counting of notochord cells. Serial cross sections in 13 μ were prepared from the embryos of external gill stage derived

from one batch. The number of the notochordal cells on each section was counted and the total number per embryo was obtained by summation. The mean of such total numbers was taken as a representative figure for each experimental lot.

It is admitted that total number thus obtained includes duplicate counts of one cell cut into two or more sections. However the range of error of this sort will remain roughly constant so far as the size and the shape of the notochordal cell are similar among lots to be compared. As a matter of fact this condition is realized in our experiment as is shown in the data under *D*, Tables 5, 6 (p. 231). Four embryos showing exceptionally slender notochord surrounded by loose mesodermal tissues were excluded from the calculation. As shown in Table 3, the difference in "cell number" of the notochord between control and sodium thiocyanate- or iodide-series is statistically insignificant at 5% level.

Table 3. The number of cells in the notochord of *Bufo vulgaris*

	Twisted notochord (NaSCN)	Control	"Enlarged" notochord (NaI)
Mean	9,415	9,577	9,455
Fiducial range	±767.3	±432.9	±175.7
t_0 observed	0.451	0.453	
Probability	Pr.>0.6	Pr.>0.6	
No. of cases	13	10	8

Series b. Counting of nuclei of notochord of thiocyanate-treated embryos. It appears necessary to supplement the data of series *a*, by the nuclear count of the notochord of the embryos which develop kyphosis. For the counting, the embryos at the early and middle tail bud stage were used avoiding very young stage in which cell divisions were still going on. As the malformation becomes externally evident only later than the tail-bud stage, and as only a fraction of the treated embryos develops notochord malformation, the following procedure was adopted to select embryos for the study. The embryos of *Rana nigromaculata* from one batch treated with sodium thiocyanate were used exclusively. When the embryos treated during the gastrulation reached the neurula stage, they were divided into three groups according to external morphology: Group A—Neurulae with a short

neural plate and large yolk plug; Group B—Neurulae with normal neural plate with smaller yolk plug; Group C—normal neurulae in which yolk plug has completely invaginated. A part of the embryos belonging to each group was fixed at the early and middle tail-bud stage and the remainders were cultured further until the external gill stage. As the group B was then found to produce a typical kyphosis in high incidence (*ca.* 95%), while in other groups no kyphosis developed, the fixed embryos belonging to the group B were used for counting the nuclei. Embryos were sectioned at 10 μ, and the nuclei visualized by FEULGEN's nuclear reaction. An estimation of the actual number was made according to ABERCROMBIE's formula (1946). As shown in Table 4, the difference in the total number of notochord nuclei between the control and thiocyanate-treated embryos is obviously insignificant at 5% level.

Table 4. Comparison of the number of nuclei in the notochord of *Rana nigromaculata*

	Early tail-bud stage (series A)		Early tail-bud stage (series B)		Middle tail-bud stage	
	Control	NaSCN-treatment	Control	NaSCN-treatment	Control	NaSCN-treatment
Observed mean	1,872.9	1,861.3	1,912.8	1,902.0	1,887.7	1,861.6
Actual mean*	1,027.8	1,021.7	1,049.9	1,046.0	1,030.5	1,021.9
t_0 observed	0.23		0.24		0.42	
Probability	Pr.>0.8		Pr.>0.8		Pr.>0.6	
No. of cases	17	12	10	15	10	14

* Actual mean was calculated by ABERCROMBIE's formula.

D. The size of notochord cells in iodide- and thiocyanate-treated embryos

As mentioned under the heading *C-a*, the comparison of the cell number of the notochord is possible only when the size and shape of the notochord cells between the kyphotic and control embryos are comparable. Some attempts were made to test this point. The information may be also interesting for understanding the mechanism of action of the chemicals.

a. In the notochord malformed by iodide the number of cells in one notochordal cross section with circular contour was counted in

15–30 cases. From the value obtained and the diameter of the notochord the average area of cell sections was calculated. Table 5 shows that no significant difference exists between the control and treated embryos in the value thus obtained.

Table 5. Area and number of the notochord cells in one cross section of *Bufo vulgaris* (mean value in 15–30 sections)

	Diameter of notochord (μ)		Number of cells		Area of cell (μ^2)	
	Control	"Enlarged" notochord (NaI)	Control	"Enlarged" notochord (NaI)	Control	"Enlarged" notochord (NaI)
Mean	273	301	18.4	30.9	2,360.8	2,304.3
Fiducial range	±21.45	±13.04	±1.85	±3.34	±118.71	±48.00
t_0 observed	7.02		9.25		0.771	
Probability	Pr.≪0.001		Pr.≪0.001		Pr.>0.4	
No. of cases	10	6	10	6	10	6

Table 6. Comparison of size of the notochord cells between kyphosis and control in the section of *Bufo vulgaris*. Size is expressed in terms of long and short axis of the ellipsoidal cells

Case No.	Cross section				Frontal section				Sagittal section			
	Long axis (μ)		Short axis (μ)		Long axis (μ)		Short axis (μ)		Long axis (μ)		Short axis (μ)	
	Cont.	Kypho.	Cont.	Kypho.	Cont.	Kypho.	Cont.	Kypho.	Cont.	Kypho.	Cont.	Kypho.
1	72.8	78.0	49.4	58.5	72.8	75.4	54.6	54.6	74.1	70.2	48.1	54.6
2	80.6	78.0	59.8	57.2	81.9	81.9	53.3	59.8	71.5	78.0	53.3	52.0
3	75.4	75.4	59.8	59.8	76.7	76.7	54.6	53.3	72.8	71.5	55.9	53.5
4	80.6	74.1	57.2	62.4	87.1	74.1	57.2	49.4	67.6	75.4	46.8	58.5
5	81.9	74.1	59.8	53.3	72.8	72.8	54.6	57.2	79.3	74.1	55.9	54.6
Mean	78.3	75.9	57.2	58.2	78.3	76.2	54.9	55.1	75.1	73.8	52.0	54.6
t_0	0.339		0.100		0.131		0.035		0.78		0.375	
Prob.	Pr.>0.7		Pr.>0.9		Pr.>0.8		Pr.>0.9		Pr.>0.9		Pr.>0.7	

Each value is the average obtained from 10 largest cells found in the sections through the anterior part of the notochord.

b. The size of the completely vacuolized notochord cells of thiocyanate-series was measured. In case of the cross section, the largest 10 cells found in five sections taken one in every fifth of the sections were used. In case of the frontal or sagittal section, the largest 10 cells found in five sections taken one in every second sections were used. By this way, difference in the measure of the cells along one particular axis will be detected. As is shown in Table 6, no significant difference in the values could be detected between the kyphotic and normal embryos.

In short, the abnormalities of the notochord caused by iodide- and thiocyanate-treatment are not associated with increase in the number or in the volume of notochordal cells within the limit of accuracy of the present tests.

Discussion

The results above presented indicate that sodium iodide affects gastrulation and neurulation: The invagination of the presumptive mesoderm and endoderm was retarded or arrested and the stretching of the neural plate diminished. The tail abnormalities such as bending and duplication may be secondary effects of primary disturbances in gastrulation caused by the treatment. On the other hand, many abnormalities in differentiation of the nervous system, such as thickening of the wall of the brain, irregular or supernormal formation of the neural canal, discharge of free cells from the neural epithelium, are not necessarily the results of disturbances in gastrulation. All malformations above enumerated in iodide-series are common to the thiocyanate-series (Ôgi, 1955) except the following points. (1) Whereas in thiocyanate-series the gastrula-stage was found to be the most sensitive of all stage studied, in iodide-series no clear difference in the sensitivity was found between the stages investigated. (2) In thiocyanate-series the notochord was very frequently twisted, but its diameter was seldom enlarged. On the contrary, in iodide-series twisting of the notochord was not observed, but local thickening of the notochord was very often met with. Peculiar to iodide-series was the branch of the notochord which penetrated the brain or the enteric roof, often forming an accessory notochord running parallel to the primary one. (3) The protuberance on the ventral or latero-ventral flank containing a mass of undifferentiated mesoderm cells was also

characteristic of the iodide-series (cf. p. 227).

In interpreting the effect of thiocyanate and iodide on the morphogenesis, it is essential to know whether or not the notochord abnormalities are associated with excess amount of the tissue. In this connection it must be emphasized that an increase in the cross-sectional area in a limited region of the notochord does not necessarily implies supernormal amount of notochordal tissue as a whole. In supernormal thickening of the notochord as the result of mechanical suppression of caudal stretching such as reported by HOLTFRETER (1945), it is highly likely that the total amount of the notochordal tissue is not affected at all.

RANZI and his coworkers are of the opinion that thiocyanate-treatment does produce supernormal amount of notochordal tissue ("ipersviluppo della corda"), on the basis of the enlargement of the notochord cross section and also on the following numerical data: (1) The total volume of the differentiated notochord of one thiocyanate-treated larva of *Rana esculenta* was found to be $86.0 \times 10^6\ \mu^3$, whereas that of one control larva $48.5 \times 10^6\ \mu^3$. (2) In the early notochord rudiment of one thiocyanate-treated embryo 1,561 nuclei were counted in contrast to 1,090 nuclei in that of one control embryo (RANZI, 1945).

The results reported in the present paper show no significant difference between the number of cells in the twisted or locally thickened notochord of the thiocyanate- or iodide-treated embryos and that of the normal notochord of control embryo, thus fail to confirm the Italian authors. Further, between the thiocyanate-series and the control, the total number of nuclei of the notochord did not differ as far as the early and middle tail-bud stages are concerned. All these facts do not support the idea that notochord abnormalities caused by thiocyanate or iodide are accompanied by an increase of the notochordal tissue at the expense of other tissues within the limit of accuracy of the method adopted by the present auther.

If the malformations of the notochord in thiocyanate- and iodide-series are not explicable in terms of supernormal formation of the notochord, what is the mechanism involved? Although it is impossible to give a definite answer, at the present stage of the study, some possibilities may be suggested. As stated above both treatments have suppressive effect on the morphogenetic movements, especially stretch-

ing. Thus it is possible that both treatments cause the notochord malformations by interfering with morphogenetic movements of the presumptive notochord. Local increase in cross section, twisting or even branching could be the results of such suppression of stretching. Another possibility might be pointed out: It has been demonstrated especially by isolation experiments that typical shape and structure of tissues and organs are established only in the presence of some other tissues or layers which are in direct contact with the formers. For instance, when the notochordal anlage is enveloped in an endodermal matrix and cultured, the notochord elongates, but its caudal stretching is not as intensive as in the intact embryo (TOWNES and HOLTFRETER, 1955). In exogastrulation the notochord is differentiated in the meso-entodermal mass. However it does not show the intensive caudal stretching characteristic for the normal notochord. If a piece of the presumptive ectoderm is attached to the caudal part of the exogastrulated chorda-mesoderm, the latter stretches into the ectoderm, induces a neural tube, and organizes a tail (HOLTFRETER, 1933). Thus the caudal stretching of the notochord appears to be under the influence of the ectoderm with which it comes into contact. In the present experiments most of the embryos with thickened or twisted notochord show various morphological abnormalities in the ectodermal organs. This can be interpreted as suggesting that the malformations of notochord are due to a disturbance in its morphogenetic movement, which is, in turn, caused by lack of adequate morphogenetic stimulus from the adjacent ectoderm which is primarily affected by the treatment.

JENKINSON (1906) produced notochordal tissue embedded in enteric roof, wall of neural tube and perichordal mesenchyme by treating the frog embryo with strong solutions of urea. Since such notochordal tissue was not necessarily connected with the primary one and frequently completely embedded in the foreign tissue, he concluded that this accessory tissue was not derived from the primary notochord. FAUTREY (1951) concluded that urea-treatment shifts the course of development of the affected cells toward the notochordal direction. In the present iodide-series the accessory notochordal tissue is directly connected with the primary notochord in all cases and hence very probably must have been derived from the primary anlage. A slight deviation of the dynamic condition from the normal might bring about

a branching of the notochord. The notochord has been found to bifurcate when it develops under some abnormal situation as in isolation (HOLTFRETER, 1936, p. 520; YAMADA, unpublished) or in transplantation (TÖNDURY, 1936, p. 53).

On the other hand, it has been found that sodium thiocyanate can sometimes initiate the differentiation of notochord or myotome in the ventral mesoderm: Pieces of the ventral marginal zone of *Triturus* gastrula were treated with M/30 sodium thiocyanate until they begin to disintegrate and then transferred to HOLTFRETER's solution. The reaggregated isolates differentiated dorsal tissues such as notochord and myotomes, whereas the untreated isolates differentiated blood cells, blood vessels, mesenchyme and nephric tubules but no dorsal tissues. The same treatment of the presumptive ectoderm of the gastrula lead to the differentiation of brain, eye and nose (ÔGI, unpublished). The results are in perfect agreement with those of ammonia-treatment of the corresponding isolates (YAMADA, 1950). Thus thiocyanate is potentially able to increase the amount of notochord and other dorsal tissues at the expense of more ventral tissues. Such effect is more evident when it is applied on the small isolate, than when applied on the whole embryo. Apparently the difference depends on the extent of penetration of chemicals into the cell interior.

SUMMARY

1. The embryos of *Bufo vulgaris* from a single batch were treated with various concentrations of sodium thiocyanate for 24 hours. Besides morphological study of treated embryos, an estimation of total cell number of the notochord was attempted. Further, the tail-bud embryos of *Rana nigromaculata*, which were treated with M/40 sodium thiocyanate for 12 hours, were also used for the estimation of total cell number.

2. By treating with sodium iodide, embryos with permanent yolk plug, double or bent tail were obtained. In the external appearance these embryos were similar to those obtained with sodium thiocyanate.

3. In iodide-treated embryos the notochord formed branches or processes, which penetrated the wall of brain or the enteric roof, often forming an accessory notochord. Also enlargement of the cross section of the notochord was often observed.

4. The enlargement of the cross section of the notochord produced by iodide and the twisting caused by thiocyanate were not accompanied by detectable increase in the total number of cells of the notochord. It is thus unlikely that these treatments cause chordalization of the cells not belonging to the presumptive notochord.

5. In iodide-treated embryos mass of undifferentiated mesodermal cells accumulated in the blood island region, sometimes forming a protuberance in the posterior abdomen. Apparently the cells represent the presumptive material of blood island inhibited in differentiation.

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THE SECRETION OF HATCHING ENZYME FROM SEA URCHIN BLASTOMERES REARED IN Ca-FREE SEA WATER*

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The sea urchin blastulae secrete an enzyme—the "hatching enzyme"—which dissolves the fertilization membrane allowing themselves to swim out into the sea water (ISHIDA, 1936; KOPAC, 1941; SUGAWARA, 1943; ENDO, 1952). Just before hatching, notable changes take place in the embryos. Not only do they secrete the enzyme but acquire cilia on the surface and moreover the blastular wall suddenly becomes impermeable to cane sugar molecules (MOORE, 1940; DAN, 1952). Since these phenomena follow so closely one upon the other, it is natural to imagine that there may be some correlation among them. As a matter of fact, ISHIDA (1952) postulated that the hatching enzyme becomes effective as a result of the ciliary beat. From the experiments to be reported, however, the author arrived at a contradictory conclusion.

EXPERIMENTS

The fertilized eggs of *Hemicentrotus* (*Strongylocentrotus*) *pulcherrimus* were washed with Ca-free sea water before the first cleavage, and allowed to remain in it. In the Ca-free sea water, although the succeeding cleavages occur with the same speed as in normal sea water, the hyaline layer is not formed, and looscly connected blastomeres fail to form the usual spherical embryos. They remain as crusters even until the control embryos begin to rotate within the fertilization membranes as blastulae, and they do not form cilia. Following cilia formation, the control embryos hatch out. At about

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the same time as the control embryos hatch out, the fertilization membranes of the Ca-free batch begin to disintegrate at one point and are gradually dissolved. Finally the blastomeres spill out from the membranes into the medium (Fig. 1).

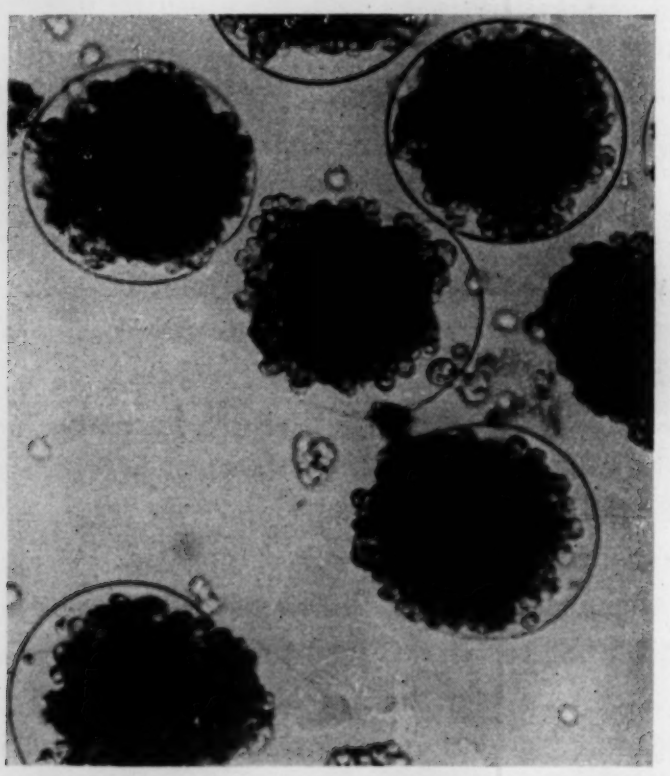


Fig. 1. Dissolution of the fertilization membranes in Ca-free sea water. ×180

Considering the time alone it can be inferred that the hatching enzyme must also be secreted from the disconnected non-ciliated blastomeres. Since the fertilization membrane is very stable in its chemical nature (E. N. HARVEY, 1910), it is hardly possible that any other agencies besides the specific enzyme can dissolve it. A test was therefore made to determine whether or not the Ca-free medium in which embryos had been reared to hatching contains the hatching enzyme. The fertilized eggs with the hardened fertilization membranes were incubated at 25° C. in this medium; after the lapse of 1 or 1½ hours, the fertilization membranes were found to have been dissolved (Fig. 2).

SUGAWARA (1942) pointed out that the hatching enzyme was inactivated by a Ca-free condition. The present results seem to contra-

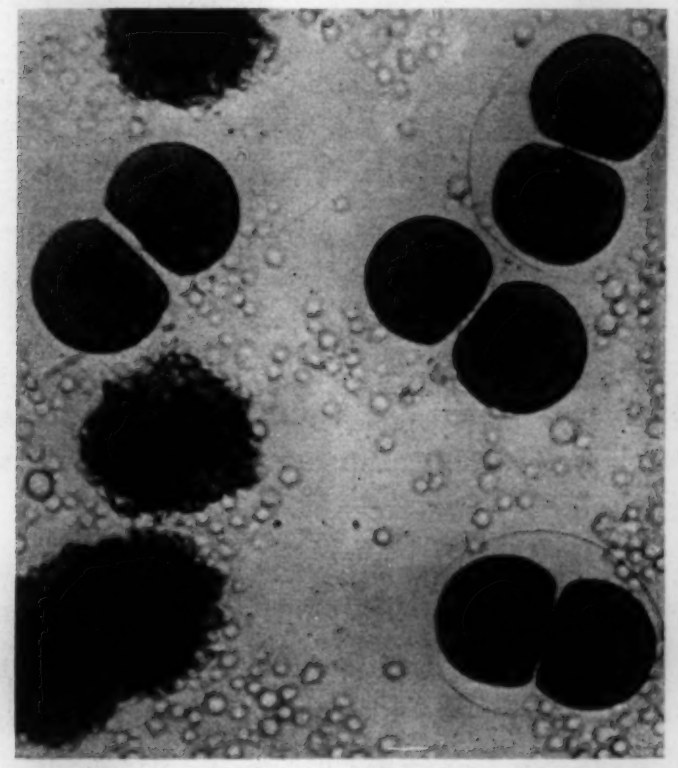


Fig. 2. Dissolution of the fertilization membranes of other eggs in the same medium as Fig. 1. ×167

dict his conclusion. There is, however, a possibility that a minute amount of calcium may have been released from the blastomeres during development which is sufficient to activate the hatching enzyme. Moreover, when fertilized eggs were transferred to the above-mentioned Ca-free medium from sea water, some calcium ions might have been introduced. The following experiment supports these inferences. The time required for dissolution of the fertilization membranes was compared under three conditions: a) fertilized eggs were transferred directly from sea water to the Ca-free medium in which embryos had been reared to hatching; b) fertilized eggs were washed several times with Ca-free sea water previous to the transferrence to the preparation; c) washed eggs were transferred to the preparation which had been added 10% by volume of isotonic solution of sodium citrate. The result is, as shown in Table 1. that the time required for the membrane dissolution becomes longer when the eggs have been washed in Ca-free sea water (b) than when unwashed (a), and the membranes do not dissolve even after 24 hours in the citrated preparation (*c*).

Table 1. Dissolution of the fertilization membranes in the supernatants incubated at 25° C.

	Ca-free supernatant		Citrated Ca-free supernatant
	a. Unwashed eggs (%)	b. Washed eggs (%)	c. Washed eggs (%)
1 hr.	100	70	0
2 hrs.		100	0
24 hrs.			0

If the blastomeres which are scattered in the medium are transferred to normal sea water, after about 30 minutes they aggregate and then acquire cilia.

Discussion

It has been asserted by Ishida (1952) that the hatching enzyme is formed or activated by the movement of the blastular cilia, on the basis of the observation that gum arabic or excess of calcium in sea water inhibits the blastular ciliary movement and simultaneously prevents the appearance of the hatching enzyme. However, the present experiments show that embryos which have been reared in Ca-free sea water do secrete the hatching enzyme in the absence of cilia. Therefore, it seems likely that the agents used by Ishida may have had effects on other than cilia.

In normal development, although the change in permeability of the blastular wall, the formation of the cilia and the secretion of the hatching enzyme follow one another closely (Dan, 1952), the results described above show that the hatching enzyme is secreted independent on other two. A rather small amount of calcium is sufficient for the activation of the hatching enzyme, compared with the amount required for adhesion of the blastomeres and formation of the cilia.

Summary

The hatching enzyme is secreted from non-ciliated blastomeres which have been reared in Ca-free sea water. That means the formation of a normal blastula and the movement of the cilia are not necessary for the formation and the secretion of the hatching enzyme.

The author's thanks are due to the director and staff of Misaki Marine Biological Station where the work was carried out.

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A STUDY OF THE ALANYLGLYCINE DIPEPTIDASE ACTIVITY DURING THE DEVELOPMENT OF *ILYANASSA OBSOLETA**

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INTRODUCTION

CONKLIN (1902, page 107) stated the problem of differentiation as follows: "In the main, differentiation in the early development of an animal consists in the formation of various unlike substances and in their localization in different regions of the egg or in different blastomeres." Such a process of localization was indicated by the earlier cell-lineage studies; following this early descriptive work the segregation of embryonic potencies was demonstrated by the isolation experiments of CRAMPTON (1896), WILSON (1904) and others. CLEMENT (1947, 1951, 1952, 1954, 1956) has repeated and extended the earlier work of CRAMPTON (1896) with the egg of *Ilyanassa obsoleta* SAY.

Having thus obtained a description of certain developmental events in terms of the localization of potencies, it is then necessary to determine what constitutes the material basis of these potencies. The centrifugation studies of MORGAN (1910), CONKLIN (1931), HARVEY (1946), and CLEMENT (1937) have shown that the degree of organization necessary for development resides in the hyaline protoplasm. Since the hyaline protoplasm cannot readily be resolved into qualitatively different types by the available methods of physical analysis,

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chemical methods have been used in an effort to obtain a more extensive description of the materials responsible for differentiation. The importance of enzymatic catalysis in cellular metabolism suggests that an analysis of the enzymatic activity of germinal regions with different developmental potencies may yield some information regarding the metabolic pathways on which differentiation is dependent.

HOLTER, LANZ, and LINDERSTRØM-LANG (1938) found an equal distribution of alanylglycine dipeptidase activity to the first eight blastomeres in the case of *Psammechinus miliaris*; further, HOLTER and LINDAHL (1941) found that there was no accumulation of dipeptidase activity in the animal or vegetal halves of the blastula, nor in the ectoderm, endoderm, or mesoderm of the gastrula of *Paracentrotus lividus.*

These results obtained with regulative eggs suggested a similar study with the mosaic egg. The following experiments represent an attempt to trace the distribution of alanylglycine dipeptidase activity to the early blastomeres and some of the germinal regions of the embryo of the gastropod, *Ilyanassa obsoleta.*

The author gratefully acknowledges his indebtedness to Professor D. P. COSTELLO for his interest and helpful guidance during the course of this study.

MATERIALS AND METHODS

During the summers of 1952 and 1953 at the Marine Biological Laboratory, Woods Hole, Massachusetts, the eggs of *Ilyanassa* were obtained from snails maintained in a laboratory aquarium. These snails were fed daily, and an abundance of egg capsules was obtained each day from the middle of June until September. During the winter months about 30 snails were kept in a five-gallon glass aquarium in the Zoological Laboratory at the University of North Carolina. These snails were fed two to three times a week, and the sea water in the aquarium was changed after each feeding. Fresh water clams served as a food source during this period. An adequate number of eggs was obtained from November until May. Eggs were obtained during the winter months by artificially increasing the daylight period, according to the procedure of JENNER (1954), to about 14 hours.

The *Ilyanassa* egg is fertilized internally and within about fifty minutes after laying, a cytoplasmic protrusion, the polar lobe, appears

at the vegetal pole just prior to the completion of the first maturation division. A second polar lobe appears during the second maturation division, and a third lobe is formed just before the formation of the first cleavage furrow and remains until the cleavage furrow is completed. At this stage the third polar lobe and the first two blastomeres appear as three distinct spheres which are approximately equal in size and are connected by protoplasmic bridges. The third polar lobe is absorbed into the *CD* blastomere at the end of the first cleavage, and as a result the *CD* blastomere is considerably larger than the *AB* blastomere. A fourth polar lobe is formed by the *CD* blastomere just prior to the second cleavage, and this lobe is absorbed into the *D* blastomere, which results in the characteristically larger *D* blastomere at the four-cell stage. The third and subsequent cleavages are essentially the same as in the case of *Crepidula.*

Blastomere isolation was accomplished by placing the eggs in calcium-free sea water (LYMANS' formula, 1940) about thirty minutes prior to cleavage. The differentiation of blastomeres isolated with calcium-low sea water was compared with the differentiation of isolates separated with a fine glass fiber, and the results were found to be the same in both cases. All embryos and isolates were reared at 20° C. in pasteurized sea water. All isolates were reared for ten days, and at this time the differentiation of each isolate was complete.

Development of Normal and Partial Embryos

By the end of the second day of development, in the case of the normal embryo, the cilia of the trochoblast, apical plate, and posterior plate cells are sufficiently developed to impart a slow rotary movement to the embryo. At this time the anlage of the shell gland is visible as a dense area in the mid-dorsal posterior region of the embryo. At about the third day the embryo begins to elongate, and by the fourth or fifth day the primordia of the shell, foot, operculum, otocyst, velum, head vesicle and external kidneys are readily recognizable in the living embryo. A fully-differentiated veliger larva is indicated schematically in Fig. 1.

The "lobeless larva" (Fig. 3) appears as a mass of incompletely-formed parts. The anterior surface has an oblong contour when viewed from above, and bears several groups of strong velar cilia. Projecting from the posterior side is a relatively large mass of endo-

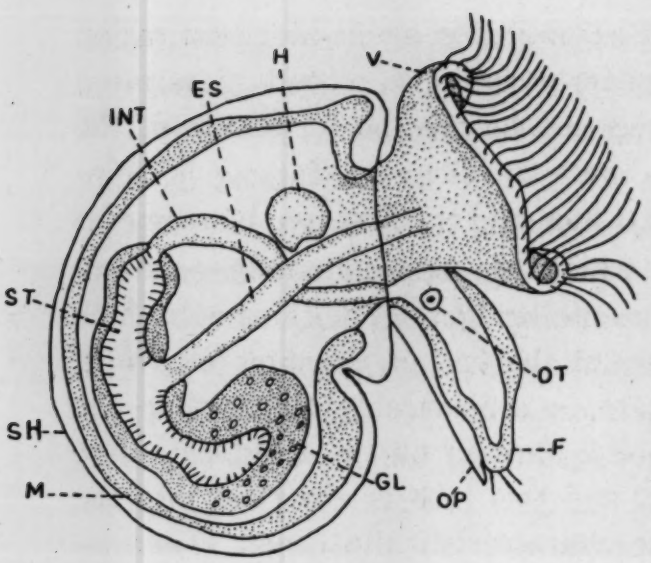


Fig. 1

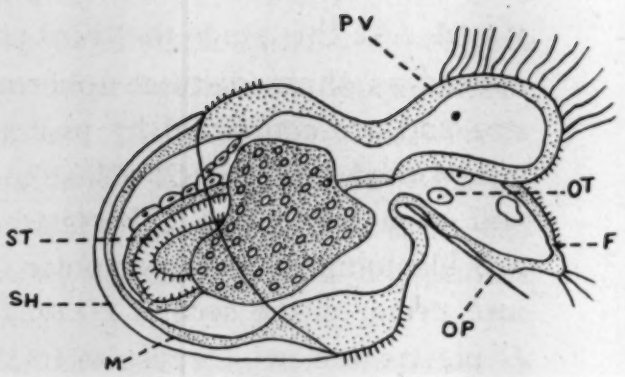


Fig. 2

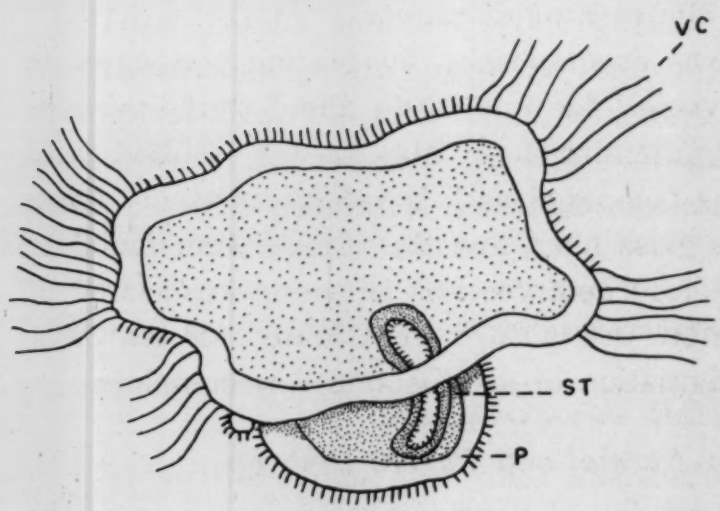


Fig. 3

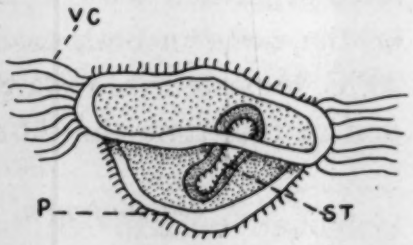


Fig. 4

Fig. 1–4. Comparison of normal and partial larvae.

Fig. 1. Normal nine-day-old veliger larva; *V*, velum; *H*, heart; *ES*, esophagus; *INT*, intestine; *ST*, stomach; *SH*, shell; *M*, mantle; *GL*, digestive gland; *OP*, operculum; *F*, foot; *O*, otocyst.

Fig. 2. Nine-day-old *CD* larva; *PV*, partial velum.

Fig. 3. Nine-day-old lobeless larva; *VC*, velar cilia; *P*, posterior protrusion.

Fig. 4. Nine-day-old *AB* larva.

dermal cells which CLEMENT (1952) has termed the "posterior protrusion." Typically, the "lobeless larva" has many large velar cilia, contractile mesenchyme cells, and a mass of endodermal cells which later form a ciliated stomach. The development of a foot, otocysts, operculum, shell, beating heart, external kidneys, fully-formed diges-

tive system, or a complete velum was not observed. Over 300 lobeless isolates were studied.

The development of 150 *AB* isolates was studied. From Fig. 4 it is seen that the ten-day *AB* larva is quite similar to the fully-differentiated, lobeless isolate in that it has several groups of velar cilia distributed along an ectodermal cap, and a "posterior protrusion" which contains many endodermal and mesenchyme cells.

The differentiation of the *CD* isolate is completed by about the ninth day. Of the 60 cases studied, no completely normal larvae were obtained. The differentiation of the endodermal structures and the velum was deficient in all cases. The general features of the fully-differentiated *CD* larva are indicated in Fig. 2. In the larva figured, the shell is somewhat reduced, although often a more complete shell has been observed. In most cases a foot with paired otocysts, operculum, heart, mantle cavity, rudimentary endodermal structures, shell, partial velum, and eye spots appeared in the nine-day *CD* larva.

Determination of the Dipeptidase Activity and the Volume of the Hyaline Protoplasm

The dipeptidase activity was determined by the method of LINDERSTRØM-LANG and HOLTER (1931, 1932). The essentials of this procedure are: (1) cytolysis of the eggs or isolates in 2 μl. of distilled water buffered to a pH of 7.4 with phosphate buffer, (2) extraction of the enzyme with 4 μl. of 60 per cent glycerol in 0.066 M phosphate buffer at pH 7.4, (3) incubation for 18 hours at 40° C. with 6 μl. of 0.2 M alanylglycine the pH of which was adjusted to 7.4 with sodium hydroxide, and (5) titration of the hydrolyzed amino acid residues with 0.05 N alcoholic hydrochloric acid (acid contained in 90 per cent ethanol) in an acetone medium containing 1 mg. per cent of naphthyl red (benzene-azo-alpha naphthylamine) as an indicator.

For the determination of the dipeptidase activity the eggs or isolates were transferred to a small reaction vessel (4 mm. in diameter and 2.5 cm. high). All determinations were done in quadruplicate and for each series four blank vessels were included. In preparing the blank vessels the eggs or isolates were omitted, since control experiments showed that the presence or absence of the eggs made no difference in the blank titration. The dipeptidase activity was determined by subtracting the titration value of the blank from the

titration value of the complete reaction mixture. In all cases the biological sample used was adjusted so that the difference between the blank titration and the enzyme preparation was about ten times greater than the sensitivity of the analytical procedure. The microliter burette of SCHOLANDER, EDWARDS and IRVING (1943) was used for the titrations; the burette used had a sensitivity of 0.15 μl.

The volume of the hyaline protoplasm was determined by subtracting the volume of the yolk and oil droplets from the total volume of the egg or blastomere. The volume of the yolk was measured by centrifuging the yolk platelets of 50 to 100 homogenized eggs into the closed end of a capillary tube and measuring the volume of the capillary occupied by the yolk platelets. The capillary containing the packed yolk platelets was mounted in a drop of immersion oil under a coverslip, and the dimensions of the capillary were measured with a filar micrometer. The volume of the non-tapering portion was calculated as the volume of a cylinder, and the volume of the tapering portion as a segment of a cone. Cases in which the volume of the capillary could not be resolved into the volume of a cylinder and/or a segment of a cone were discarded. In all cases seven or eight determinations were made, and the mean value was taken as the volume of the yolk. The standard error of the mean was equal to 8 per cent of the value for the volume of the yolk in the case of the measurements of the whole egg, and the size of the sample in all other cases was adjusted so that the total volume of the yolk was approximately equal to the total volume of the yolk in the whole egg. The volume of the oil droplets was determined by measuring the dimensions of the oil cap in the intact centrifuged egg and calculating the volume as a spherical segment.

RESULTS

1. Intracellular Localization of Dipeptidase Activity in the Whole Egg. The *Ilyanassa* egg was fragmented into two nearly-equal halves by centrifuging the egg in gum arabic for 10 minutes at a centrifugal force of about 2,000 times gravity, and the dipeptidase activity of each fragment was determined. Before measuring the dipeptidase activity in the centrifuged fragments, the diameter of the centripetal fragment was measured after it had become spherical, and the volume was calculated as a sphere. The volume of the centrifugal fragment

could not be measured directly as these fragments seldom assumed a spherical form after centrifugation; however, the volume of the centrifugal half was obtained by subtracting the volume of the centripetal half from the volume of the whole egg. Since all the yolk was displaced centrifugally and the oil cap centripetally, the data for the whole egg was used in arriving at the volume of these constituents in the two fragments. The volume of the hyaline protoplasm of each fragment was obtained by subtracting the volume of the oil cap, in the case of the centripetal half, and the volume of the yolk, for the centrifugal half, from the total volume of each fragment.

Table 1. Dipeptidase activity and the volume of the hyaline protoplasm of the egg fragments
Volume as 10^{-4} mm.^{3}

Material	Total	Yolk	Oil Cap	Hyaline protoplasm	Dipeptidase activity*	Activity in %
Whole egg	21.8	7.0	1.5	13.3	0.33	100
Centripetal fragment	11.0	0.0	1.5	9.5	0.23	69.6
Centrifugal fragment	10.8	7.0	0.0	3.8	0.07	21.2

* Expressed as microliters of 0.05 N HCl.

From the data presented in Table 1, it is seen that 69.6 per cent of the total dipeptidase activity was found in the centripetal half, and 21.2 per cent in the centrifugal half. This represents a recovery of 90.8 per cent of the activity of the intact egg. The failure to obtain complete recovery was probably due to the loss of some activity from the centrifugal half, since this fragment, in marked contrast to the centripetal fragment, always appeared somewhat damaged. These values or the percentage distribution of dipeptidase activity are closely correlated with the percentage distribution of the hyaline protoplasm, as listed in the last three rows of Table 1.

Since the hyaline zone of the centripetal half contained the mitochondria, the localization of dipeptidase activity on these particulates remains as a possibility; however, this seems unlikely in view of the fact that no mitochondria were observed in the centrifugal fragment which contained 21.2 per cent of the activity. It is therefore concluded that the alanylglycine dipeptidase activity is contained in the hyaline protoplasm and/or its finer inclusions. Since the microsomes were

not displaced by the centrifugal force employed, these particulates must be considered together with the hyaline protoplasm in this study.

2. Dipeptidase Activity of the Whole Egg, Third Polar Lobe, Lobeless Egg, AB, CD, C, and D Blastomeres. The dipeptidase activity of the egg and various isolates was determined. The diameter of the whole egg and the isolates was measured with an ocular micrometer and the volume was calculated as the volume of a sphere. In the case of the dipeptidase determinations of the polar lobe and ioslated blastomeres, each series of determinations was made from eggs and isolates from the same egg capsule, and the sum of the activity of the isolates was compared with the activity of the whole egg. Those cases in which the sum of the activity of the parts varied more than 10 per cent from the activity of the whole were discarded. Five to ten eggs or isolates were used per determination, except in the case of the polar lobe, in which twenty-five were used.

Table 2. Volume data for the egg and blastomeres
Volume expressed as 10^{-4} mm.^{3}

Material	Total	Yolk	Oil	Hyaline protoplasm
Egg	21.8	7.0	1.5	13.3
Third polar lobe	4.9	2.7	0.05	2.2
Lobeless egg	16.9	4.3	1.4	11.2
AB blastomere	7.6	1.5	0.5	5.6
CD blastomere	13.4	4.7	1.0	7.7
C blastomere	4.0	1.3	—	2.7
D blastomere	9.2	3.9	—	5.3
Mesentoblast cells	14.4	7.0	—	7.4
Ectoblast cells	7.4	0.0	—	7.4

The volumes of the eggs and isolates are presented in Table 2; each value listed represents the mean of eight measurements.

The data for the dipeptidase activity of the whole egg, third polar lobe, lobeless egg, *AB*, *CD*, *C*, and *D* blastomeres are presented in Table 3. A comparison of the data listed in columns 3 and 4 in Table 3 shows a striking parallel between the distribution of the dipeptidase activity and the distribution of the hyaline protoplasm in the polar lobe, lobeless egg, and isolated blastomeres. It is seen from column

4 of Table 2 that there are no data available for the volume of the oil in the *C* and *D* blastomeres. However, by using the data for the volume of oil in the *CD* blastomere, which is divided between the *C* and *D* blastomeres, the magnitude of the error involved can be estimated. Assuming that the distribution of the oil to the *C* and *D* blastomeres follows the same percentage distribution as in the case of the *AB* and *CD* blastomeres, this error would then reduce the values obtained for the volume of hyaline protoplasm for the *C* and *D* blastomeres by 11.1 and 13.2 per cent, respectively. This error in the volume of the hyaline protoplasm results in an alteration of the values for the percentage distribution of the hyaline protoplasm to 18.0 per cent for the *C* and 34.6 per cent for the *D* blastomere. While this tends to decrease the correlation between the distribution of the hyaline protoplasm and the dipeptidase activity, these two values are still in fairly close agreement.

Table 3. Alanylglycine dipeptidase activity of the egg, third polar lobe, lobeless egg, *AB*, *CD*, *C* and *D* blastomeres

Material investigated	Activity per egg or isolate[1]	Dipeptidase activity in per cent of the activity of the whole egg	Volume of hyaline protoplasm in per cent of the volume of the whole egg
Egg (60/300)[2]	0.33±0.01	100	100
Third polar lobe (19/280)	0.05±0.01	15.1	16.5
Lobeless egg (15/120)	0.29±0.01	87.0	84.2
AB blastomere (23/215)	0.11±0.01	33.3	42.1
CD blastomere (26/230)	0.21±0.01	63.7	57.8
C blastomere (11/120)	0.09±0.01	27.2	20.3
D blastomere (17/160)	0.14±0.01	42.4	39.8

[1] Enzymatic activity expressed as microliters of 0.05 N HCl with the standard error of the mean.

[2] Figures in parentheses are number of isolates/number of determinations.

In the determination of the values for the volume of the hyaline protoplasm listed in Table 2, the volume of the mitochondria was not accurately measured, because it was not possible to determine what fraction of the mitochondria occupied the interstices between the

rather large yolk platelets. Another source of error involved in the estimation of the volume of the hyaline protoplasm was due to the fact that the yolk of the *Ilyanassa* egg is composed of platelets of different sizes and shapes, which results in the presence of interstitial spaces when these platelets are packed in the capillary tubes used for measuring the yolk volume. Since the platelets do not form stratified layers of different sized platelets when they are centrifuged in sea water, it was not possible to arrive at an accurate estimate of the error due to this packing effect. However, this error is (1) a constant factor for all of the measurements made, and (2) partially offset by the infiltration of mitochondria and small yolk granules into some of the interstitial spaces.

On the basis of these results and the foregoing study of the intracellular localization of this enzyme, it is concluded that, in the cases studied, the alanylglycine dipeptidase activity is distributed to the blastomeres and/or egg fragments in proportion to the volume of the hyaline protoplasm.

3. The Dipeptidase Activity of the Ectoblast and Mesentoblast Cells. After the completion of the seventh cleavage, which occurs within 4.5 to 5 hours after the formation of the third polar lobe, the *Ilyanassa* egg has divided into 24 cells. At this stage there are four large yolk-laden cells, the macromeres, which are called the mesentoblast cells, and it is from these cells that almost all of the mesodermal cells and all of the endodermal cells of the future embryo are derived. The remaining twenty blastomeres are small yolk-free cells, the micromeres, which cover the animal pole surface of the mesentoderm cells. From these twenty micromeres all the ectodermal cells of the future embryo will be derived; they are therefore designated as the ectoblast cells, in contrast to the mesentoblast cells. The twenty micromeres at the animal pole are not entirely ectodermal, because some scattered mesodermal cells, the "larval mesoblast cells," are derived from at least three of these micromeres. However, the ectodermal and endodermal protoblasts are completely segregated at this stage.

In order to determine the distribution of alanylglycine dipeptidase activity at this stage, the protoblasts of the ectoderm were isolated from the mesentoblast cells by destroying and removing the 3*A*, 3*B*, 3*C*, and 3*D* macromeres with a fine glass needle. The intact ecto-

blast cells were then transferred through several changes of sea water. Ten isolates were used for each determination of the dipeptidase activity. The activity of the entire 24-cell stage was also determined on a sample of eggs from the same capsule as that from which the ectoblast cells were obtained. The activity of the mesentoblast cells was calculated by subtracting the activity of the ectoblast cells from the activity of the 24-cell stage.

The procedure of puncturing the macromeres introduced the possibility of contamination of the isolated ectoblast cells with cellular debris. As a control for this error, several isolates of ectoblast cells were placed with more cytolyzed macromeres than were present in the operating dish during the isolation of the micromeres. In no case did this treatment result in a significant difference in the dipeptidase activity of the micromeres.

The volume of the ectoblast cells was determined by isolating each blastomere as it was formed, and measuring its diameter. The volume of the mesentoblast cells was obtained by subtracting the volume of the ectoblast cells from the volume of the whole egg.

The data for the volume of the hyaline protoplasm of the ectoblast and mesentoblast cells are presented in Table 2. The volume of the yolk of the mesentoblast cells was taken to be equal to the volume of yolk in the whole egg, since nearly all the yolk is contained in these cells at the 24-cell stage; the volume of yolk in the ectoblast cells is therefore negligible. It was not possible to estimate the volume of the oil droplets of the mesentoblast and ectoblast cells.

Table 4. Alanylglycine dipeptidase activity of the ectoblast and mesentoblast cells

Material	Activity per isolate[1)]	Dipeptidase activity in per cent of the activity of the whole egg	Volume of hyaline protoplasm in per cent of the volume of the whole egg
Ectoblast cells (9/85)[2)]	0.14±0.01	42.4	55.6
Mesentoblast cells	0.19±0.01	57.5	55.6

[1)] Enzymatic activity expressed as microliters of 0.05 N HCl with the standard error of the mean.

[2)] Figures in parentheses are number of isolates/number of determinations.

From the data presented in Table 4, it is seen that the percentage distribution of the dipeptidase activity to the ectoblast and mesentoblast cells is correlated with the distribution of the hyaline protoplasm.

4. Dipeptidase Activity of the Whole Embryo, Lobeless, AB and CD Isolates during Development. In order to determine the relation between the initial amount of activity allocated to any given isolate and the activity throughout the course of development, the dipeptidase activity was measured at 24-hour intervals during the development of the whole embryo, and of the lobeless, *AB*, and *CD* isolates.

Table 5. Change in alanylglycine dipeptidase activity of the normal, lobeless, *AB* and *CD* embryos during development. Activity expressed as microliters of 0.05 N HCl with the standard error of the mean

Age in Days	Normal embryo	Lobeless embryo	*AB* embryo	*CD* embryo
0	0.33±0.01 (60/300)*	0.29±0.02 (15/120)*	0.11±0.01 (23/215)*	0.21±0.01 (26/230)*
1	0.45±0.01 (38/90)	0.32±0.01 (12/60)	0.12±0.02 (9/79)	0.18±0.01 (13/65)
2	0.39±0.01 (34/170)	0.27±0.01 (10/50)	0.08±0.01 (9/84)	0.17±0.02 (11/80)
3	0.54±0.01 (27/109)	0.29±0.01 (12/60)	0.18±0.01 (12/60)	0.24±0.01 (13/65)
4	0.71±0.02 (20/105)	0.60±0.04 (19/81)	0.27±0.02 (8/60)	0.44±0.01 (13/74)
5	1.34±0.03 (26/70)	0.82±0.05 (21/79)	0.38±0.02 (14/73)	0.60±0.02 (19/65)
6	1.76±0.05 (19/57)	1.66±0.10 (23/72)	0.66±0.04 (23/97)	0.92±0.03 (17/57)
7	2.16±0.05 (38/117)	1.65±0.05 (56/140)	0.56±0.03 (19/71)	1.17±0.04 (16/50)
8	2.11±0.03 (41/121)	1.47±0.06 (32/97)	0.63±0.04 (14/56)	1.37±0.05 (19/38)
9	2.19±0.12 (35/105)	1.33±0.08 (24/76)	0.63±0.02 (12/41)	— —

* Figures in parentheses are number of isolates/number of determinations.

The mean value for the dipeptidase activity with the standard error of the mean is presented in Table 5 for all isolates. In Fig. 5, the activity is plotted against time in days. From this plot it is

seen that the activity of the normal embryo increases slightly after 24 hours, and by the end of the second day, has decreased to the original level. Although these differences are statistically significant, it is difficult to be convinced of the reliability of such a small difference, chiefly because the embryos used for these determinations were not from the same egg capsule.

Between the third and fourth days of development, a definite increase in the dipeptidase activity of the normal embryo was found; and by the seventh day, a 635 per cent increase had occurred. After the seventh day, the dipeptidase activity remained nearly constant until the ninth day, which was the oldest stage studied. This may be related to the fact that the veliger is completely developed at this stage and the period of rapid growth has ended. The absence of food material in the culture dishes may also have been a contributing factor.

From the graph presented in Fig. 5, it is seen that a similar increase in the dipeptidase activity occurred during the course of the development of the lobeless, *AB* and *CD* embryos. The pattern of activity for the lobeless and *AB* embryos is very similar, except for a difference in the magnitude of the activity. A sudden leveling off with a subsequent decrease in the activity occurred in both of these isolates following the sixth day of development. The pattern of dipeptidase activity for the normal and *CD* embryos was also quite similar, and differed from that of the lobeless and *AB* embryos by the continued increase in activity up to the seventh day in the normal embryo and to the eighth day in the *CD* embryo. These differences are probably due to the fact that the lobeless and *AB* embryos have obtained their maximum growth by the sixth day while the normal and *CD* embryos have not.

The lobeless larva is morphologically abnormal in most respects (Fig. 3) having only a ciliated stomach chamber and velar cilia in common with the normal veliger. Yet the levels of the dipeptidase activity of the normal and of the lobeless embryo are strikingly similar. On the other hand, the differentiated *CD* embryo resembles the normal veliger much more than does the lobeless embryo (Figs. 1, 2, and 3), but this relationship is reversed with respect to the pattern of the dipeptidase activity (Fig. 5). The differentiation of the *AB* embryo is almost identical with that of the lobeless embryo; yet

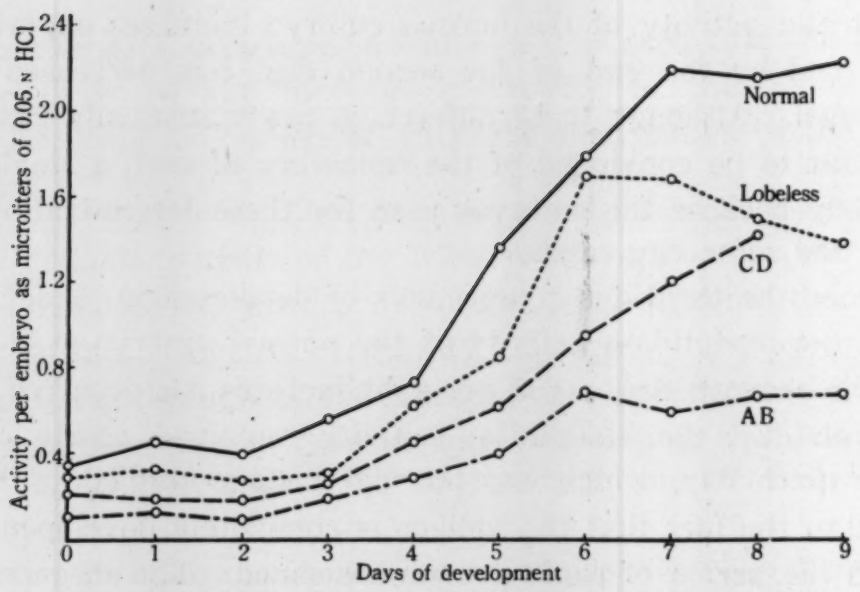


Fig. 5. Dipeptidase activity of the normal, lobeless, *CD* and *AB* embryos of *Ilyanassa Obsoleta.*

the dipeptidase activity of these two isolates are quantitatively dissimilar. If this enzyme were directly related to the segregation of morphogenetic potencies, its activity in the normal and *CD* embryos would be expected to be more closely related, and the activity of the lobeless embryo should approximate that of the *AB* embryo.

The three isolates studied have a partially developed digestive system; thus, the possibility remains that the dipeptidase activity is related to the differentiation of the digestive system. However, this appears unlikely when it is considered that the digestive system is better developed in the *CD* embryo than in the lobeless embryo, yet the dipeptidase activity of the *CD* embryo is less than that of the lobeless embryo. This point was approached more directly by rearing the isolated ectoblast cells for four days and measuring their dipeptidase activity. It was found that the activity of the four-day-old ectoblast cells represented 50.7 per cent of the activity of the whole embryo. It therefore seems unlikely that the dipeptidase activity is confined to the digestive tract. These data also indicate that the dipeptidase activity is not solely concerned with the utilization of the yolk platelets. Moreover, measurements of the volume of the yolk of the egg and of the fully-differentiated embryo at sixth day showed that no decrease in the volume of the yolk had occurred. However, a more refined method, such as the determination of the

nitrogen content of the yolk, is needed before the problem of yolk utilization can be considered with any degree of confidence.

By comparing Figs. 1 through 4, which are scale drawings of the normal, lobeless, *AB* and *CD* embryos, with the curves for the dipeptidase activity of these embryos presented in Fig. 5, it is seen that there is a direct correlation between the size of the embryos and the magnitude of the dipeptidase activity. This parallel suggests that the alanylglycine dipeptidase activity is related to growth; however, this is a difficult point to demonstrate conclusively because there is no single criterion which can be used to measure the rate of growth. Some measurements of the volume of the normal embryo were made by compressing the embryo in a capillary tube and calculating the volume from the dimensions of the compressed embryo. From these measurements, which were extended until the fifth day of development, it was found that the first significant increase in the total volume began between the third and fourth days of development. It was not possible to continue these measurements beyond the fifth day because of the appearance of the shell, which interfered with the manipulation of the embryo in the capillary tube. Although a fairly good agreement was found between the volume changes and the dipeptidase activity, it was not possible to determine whether the volume increment was due to an increase in the amount of protoplasm or to the intake of water.

Discussion

The results obtained in this study indicate that no localization of alanylglycine dipeptidase activity occurs during the development of *Ilyanassa obsoleta*, and that this enzyme does not function as a differential factor in the differentiation of the isolates studied. The data show that the dipeptidase activity is distributed to the *AB*, *CD*, *C* and *D* blastomeres, the third polar lobe, the ectoblast and mesentoblast cells in proportion to the volume of the hyaline protoplasm contained in each of the isolates. The correlation between the dipeptidase activity and the volume of the hyaline protoplasm indicates that a measure of the dipeptidase activity can be accepted as a reliable reference unit for estimating the relative amount of hyaline protoplasm in the blastomeres of the *Ilyanassa* egg. The desirability of employing an enzymatic method for measuring the volume of the

hyaline protoplasm is apparent when the difficulties and errors involved in direct determination of this volume are considered.

These results, in regard to the segregation of the alanylglycine dipeptidase activity, are in agreement with the results obtained by BERG (1954) for the distribution of alanylglycine and leucylglycine dipeptidase activity to the first two blastomeres of the egg of *Mytilus edulis*. In contrast to the marked increase in the alanylglycine dipeptidase activity in the development of *Ilyanassa*, BERG found that the activity of this enzyme remained constant during the development of *Mytilus*. The volume of the *Ilyanassa* egg is about 15 times that of the *Mytilus* egg, yet they both show the same dipeptidase activity; thus, the increase of dipeptidase activity during the development of *Ilyanassa* is probably related to the fact that the initial activity of this egg is relatively low in comparison with the activity of the *Mytilus* egg. On the basis of the available data it is not possible to decide whether these results indicate a fundamental difference in the metabolic pattterns of pelecypod and gastropod eggs.

The rapid increase in dipeptidase activity which begins between the third and fourth days of development in *Ilyanassa* is probably related to the fact that the embryo begins to increase in size at this time. This size increment is strikingly evidenced by the enlargement of the primordia of the foot, velum, and head vesicle. The relation of dipeptidase activity to growth in the chick embryo is indicated by the results of LEVY and PALMER (1940), in which the dipeptidase activity was found to be proportional to the wet weight and total nitrogen content during the development of the chick embryo. Further, the work of PATTERSON, DACKERMAN and SCHULTZ (1949) with the salivary glands of *Drosophila* indicated that the dipeptidase activity is directly related to growth. In this material, in which growth occurs without cell division, it was found that the dipeptidase activity increases at a rate proportional to the increase in the total nitrogen content. Whether the dipeptidase activity remains proportional to the amount of hyaline protoplasm throughout development cannot be established from the data available at present. If such a proportionality could be established, it would provide a method of following the synthesis of the hyaline protoplasm during growth and development.

The increase in the dipeptidase activity during the development of *Ilyanassa* should not necessarily be interpreted as indicating a syn-

thesis of this enzyme, because the mass distribution of an enzyme cannot be inferred from its activity. The work of LUNDBLAD (1954) presents an alternative explanation for such an increase in enzymatic activity. He found that the proteolytic activity of frozen-dried preparations of the eggs of *Paracentrotus lividus* was greater after treatment with crystalline ribonuclease. The possibility thus exists that the increase in dipeptidase activity, found during the development of *Ilyanassa*, results from a similar release mechanism.

SUMMARY

1. The volume of the yolk, oil droplets, and hyaline protoplasm was estimated in the egg, *AB*, *CD*, *C* and *D* blastomeres, the third polar lobe, the lobeless egg, the ectoblast and mesentoblast cells of the *Ilyanassa* embryo.

2. The alanylglycine dipeptidase activity in the above blastomeres and germinal regions was determined and it was found that this enzyme is distributed in proportion to the volume of the hyaline protoplasm.

3. The dipeptidase activity was determined during the course of development at 24-hour intervals in the normal, lobeless, *AB*, and *CD* embryos. A comparison of the dipeptidase activity and the course of differentiation indicated that this enzyme is not related to the segregation of developmental potencies.

4. An increase of over 600 per cent in the dipeptidase activity was found to occur during the development of the *Ilyanassa* embryo. The onset of this increase in dipeptidase activity was correlated with a period of rapid growth of the organ primordia which begins at about the fourth day of development.

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ANTAGONISTIC EFFECTS OF SOME CHEMICALS ON THE INDUCTION OF PARTHENOGENETIC DEVELOPMENT OF THE SEA URCHIN EGG BY HYPERTONIC TREATMENT

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It has been well-known since LOEB's classic work that although artificial formation of the fertilization membrane induces nuclear and cytoplasmic changes in the sea urchin egg, cell division does not ensue in most cases and a process of disintegration starts in a few hours. One of the effective methods for saving eggs from such disintegration is subjecting them to hypertonic treatment. If eggs are treated with hypertonic medium for an appropriate time after the membrane formation and then transferred to normal sea water, their cleavage and further development are secured. It may be considered that the hypertonic treatment induces some changes in protoplasmic condition which allows to divide and develop further.

Up to the present time, there have been two different opinions regarding the effect of hypertonic sea water; the one which sets importance on the induction of the cytaster, being based on a morphological aspect (WILSON, 1925) and the other one which insists, from a physiological standpoint, that hypertonic medium acts as an inhibitor, "corrective factor," of oxidative disintegration of the egg (LOEB, 1913).

The present study was undertaken to analyse the effect of the hypertonic treatment from a biochemical viewpoint. Experiments were carried out using dinitrophenol, sodium azide and potassium cyanide, and it was found that these reagents act in antagonism to hypertonic sea water.

MATERIAL AND METHODS

Eggs of the sea urchin, *Hemicentrotus pulcherrimus*, were used as material. The following method was adopted to induce artificial

parthenogenesis. Unfertilized eggs were treated with 6 per cent butyric acid sea water (6 cc. of N/10 butyric acid plus 94 cc. of sea water) for 50 seconds. Upon returning them to normal sea water, the membrane formation took place. After 15 minutes, they were immersed in hypertonic sea water (8 cc. of 2.5 N NaCl plus 50 cc. of sea water) for 20 to 60 minutes, and then were returned again to normal sea water.

Effects of 2, 4-dinitrophenol (DNP), sodium azide and potassium cyanide were tested. The hypertonic sea water used for these experiments were prepared by adding 8 cc. of 2.5 N NaCl solution to 50 cc. of sea water in which one of these chemicals was dissolved.

Experimental Results

1. Experiments with the hypertonic sea water containing DNP

15 minutes after artificial membrane formation by means of butyric acid, the eggs were distributed over the following solutions; (1) 8 cc. of 2.5 N NaCl plus 50 cc. of sea water, (2) 8 cc. of 2.5 N NaCl plus 50 cc. of 10^{-4} M DNP sea water. After an appropriate time they were washed with normal sea water.

The eggs treated with the former solution showed cleavage and development comparable to fertilized eggs. On the other hand, the

Table 1. Effect of DNP on the efficiency of the hypertonic treatment in parthenogenetic activation of the egg

Time of immersion (min.)	Percentage of developed eggs					
	(1)*		(2)**		(1)* after 50 minutes' treatment with (2)**	
	2-cell stage	Blastula	2-cell stage	Blastula	2-cell stage	Blastula
20	0	0	0	0	0	0
30	44	4	0	0	38	3
40	58	4	0	0	48	5
50	64	15	0	0	59	13
60	72	35	0	0	63	28

After membrane formation, eggs were treated with the following hypertonic media;

* (1) 8 cc. of 2.5 N NaCl plus 50 cc. of sea water.

** (2) 8 cc. of 2.5 N NaCl plus 50 cc. of 10^{-4} M DNP sea weter.

eggs treated with the latter failed to cleave. In these eggs, although the monaster was formed, protoplasmic disintegration followed before long just as in eggs treated with butyric acid sea water alone. The question arises as to the possibility that DNP might impair the eggs irreversibly during the hypertonic treatment. In order to examine this, the following experiment was carried out. Eggs which had been once treated with butyric acid sea water and hypertonic DNP sea water were immersed once more in hypertonic solution without DNP. It was found that they initiated cleavage in fairly high percentage. This means that the effect of DNP is not an irreversible impairment but a suppression. One of the results is shown in Table 1.

2. Experiments with hypertonic sea water containing sodium azide

Similar experiments were performed using sodium azide instead of DNP. A 5×10^{-3} M sodium azide solution in sea water was prepared and its pH was adjusted to 7.3 with N/10 HCl. Hypertonic sea water was made up in the same way as in the foregoing experiments.

15 minutes after artificial membrane formation, the eggs were put into hypertonic sea water containing sodium azide. They were returned to normal sea water after 20 to 60 minutes. No cleavage occurred. However, when such eggs were treated with hypertonic sea water without azide and then returned to normal sea water, they initiated cleavage as shown in Table 2.

Table 2. Effect of sodium azide on the efficiency of hypertonic treatment in parthenogenetic activation of the egg

Time of immersion (min.)	Percentage of cleaved eggs		
	(1)*	(2)**	(1)* after 50 minutes' treatment with (2)**
20	28	0	10
30	41	0	38
40	60	0	56
50	74	0	58
60	38	0	21

After membrane formation, eggs were treated with the following hypertonic media;

* (1) 8 cc. of 2.5 N NaCl plus 50 cc. sea water.

** (2) 8 cc. of 2.5 N NaCl plus 50 cc. of 5×10^{-3} M NaN_3 sea water (pH 7.3).

3. Experiments with hypertonic sea water containing potassium cyanide

Loeb (1906) found that the effect of hypertonic solution is completely suppressed if oxidation in the egg is inhibited by means of potassium cyanide. In the present work, therefore, experiments were performed using hypertonic sea water which contained potassium cyanide in order to compare the effect of this reagent with those of DNP and sodium azide. The results obtained coincided quite well with Loeb's. One of them is shown in Table 3.

Table 3. Effect of potassium cyanide on the efficiency of hypertonic treatment in parthenogenetic activation of the egg

Time of immersion (min.)	Percentage of cleaved eggs		
	(1)*	(2)**	(1)* after 50 minutes' treatment with (2)**
20	2	0	0
30	20	0	22
40	52	0	50
50	64	0	58
60	56	0	48

After membrane formation, eggs were treated with the following hypertonic media;

* (1) 8 cc. of 2.5 N NaCl plus 50 cc. sea water.

** (2) 8 cc. of 2.5 N NaCl plus 50 cc. of 10^{-4} M KCN sea water.

Discussion

As described above, the treatment of eggs with hypertonic sea water after artificial membrane formation becomes ineffective to induce parthenogenetic development, if the hypertonic sea water contains a small amount of DNP, sodium azide or potassium cyanide respectively. After removal from such solutions, the eggs begin to form a monaster in the cytoplasm, but do not cleave. It has also been shown that if these eggs are treated subsequently with hypertonic sea water without any of the above-mentioned inhibitors, they begin to cleave and develop. It may be inferred from these results that the chemicals mentioned above cancel the effectiveness of hypertonic sea water without impairing the egg.

According to CLOWES and KRAHL (1936) and KRAHL and CLOWES (1936, 1938), DNP causes simultaneously a stimulation of oxygen consumption and inhibition of cleavage of sea urchin eggs. ABELSON (1947) and VILLEE and others (1949) found that oxidative phosphorylation in the cell-free system and in the intact sea urchin egg is blocked by DNP. It was demonstrated by CLOWES and KRAHL (1940) that reversible inhibition of cleavage of the sea urchin egg occurs at an azide concentration of 5×10^{-3} M, inhibiting respiration by 50 per cent. Azide also is known as one of the uncoupling agents of oxidative phosphorylation in the cell-free system of the animal tissue (LOOMIS and LIPMANN 1949).

Since WARBURG (1910) demonstrated that oxygen consumption of eggs is reduced and cleavage is blocked by cyanide at a concentration of 10^{-4} M, studies on effects of cyanide upon oxygen consumption and cleavage in sea urchin eggs have been reported by a number of investigators (LOEB and WASTENEYS, 1911, 1913; BARRON and HAMBURGER, 1932; CLOWES and KRAHL, 1940; ROBBIE, 1946). Up to the present time, cyanide is widely known as a respiratory poison which inhibits the activity of cytochrome oxidase.

Therefore, it may be considered that under the influence of the chemicals the hypertonic treatment is not capable to induce parthenogenetic development in consequence of the disturbance of phosphorus metabolism which participates in energy-generating processes, such as respiration. In view of these considerations, it may be permissible to postulate that the effect of hypertonic treatment is partly at least associated with the process which involves oxidative phosphorylation.

SUMMARY

The treatment of eggs of *Hemicentrotus pulcherrimus* with hypertonic sea water after artificial membrane formation becomes ineffective to induce parthenogenetic development, if the hypertonic sea water contains a small amount of DNP, sodium azide or potassium cyanide respectively. Such an effect of the chemicals is reversible.

It is suggested that these chemicals may act on some process which is induced by the hypertonic treatment and presumably involves oxidative phosphorylation.

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QUANTITATIVE ANALYSES OF LACTIC ACID AND RELATED COMPOUNDS IN SEA URCHIN EGGS AT THE TIME OF FERTILIZATION

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INTRODUCTION

Since the work by PEARLZWEIG and BARRON (1928), many workers have studied quantitatively the change in lactic acid content of sea urchin eggs after fertilization (RAPKINE, 1931; VAN HERK, 1933; RUNNSTRÖM, 1933; ÖRSTRÖM and LINDBERG, 1940; LINDBERG, 1943 and YČAS unpublished). It is generally conceded that no change occurs (cf. ROTHSCHILD, 1951).

These studies, however, have seldom been made earlier than 10 minutes after mixing eggs and sperm, so that a re-examination of this problem seems to be necessary to clarify the quantitative change of the acid at shorter intervals after insemination.

The present paper deals with results of such an investigation. As shown in the following pages the results obtained were rather different from those of the previous authors, namely a transient production of lactic acid during the fertilization reaction was ascertained.

As a result of this finding, the effect on lactic acid production of some reagents known as inhibitors of glycolysis was studied. Quantitative analyses of related compounds during fertilization reaction were also undertaken. Here too results different from those of other workers were obtained.

MATERIALS AND METHODS

Eggs of the Japanese sea urchins, *Hemicentrotus pulcherrimus*, *Heliocidaris classispina* and *Pseudocentrotus depressus* were used. Most work was done with the eggs of *Hemicentrotus*. Only experiments using this species will be quoted, since the results obtained with other

sea urchins were always similar.

Spawning the eggs was induced by KCl solution (5/9 M) and the jelly coat was removed by acid sea water (pH *ca.* 4) followed by repeated washings. In all experiments described in this paper, more than 95% of eggs elevated fertilization membranes and cleaved normally when inseminated.

The material was prepared for analysis as follows. The eggs were packed in a hand centrifuge, the sea water removed, a known volume of 10% trichloroacetic acid added, and the suspension then homogenized in a POTTER-ELVEHJEM type homogenizer. In the case of pentose estimation, fixation and homogenization were carried out in an ice bath to eliminate pentose nucleic acid. Meta-phosphoric acid (5%) was used to fix the material prior to determination of reducing sugar. Following centrifugation (3,500 r.p.m. for 20 mins.) aliquots were taken for analyses.

Lactic and pyruvic acids were respectively estimated by the method of BARKER and SUMMERSON (1941), and of FRIEDEMANN and HAUGEN (1943) with some modifications by SHIMIZU (1950).

For the estimation of glycogen, jelly free eggs were hydrolyzed in 40% KOH solution, and the precipitate obtained by the addition of absolute alcohol to a final concentration of 60% was analyzed by the method of BOETTIGER (1946). The method described by LINDBERG (1943) was also used.

Reducing sugar was determined by the method of FOLIN and MALMROS (1929), pentose by the method of MEIJBAUM (1939) and of KERR and SERAIDARIAN (1945).

Acid hydrolyzable carbohydrate was determined by the method of MORRIS (1948) using DREYWOOD's anthrone reagent. Hydrolysis for 8 hours with 2 N HCl in a boiling water bath was sufficient to liberate all the reactive material.

Total nitrogen was determined by direct Nesslerization of H_2SO_4/H_2O_2 digests, and protein N was taken as that precipitable by 10% trichloroacetic acid.

All colour determination were made with a Hitachi electrophotometer.

All experiments were performed at room temperature (*Hemicentrotus* 18–20° C., *Heliocidaris* 25–27° C., *Pseudocentrotus* 15–17° C.).

Results

Lactic acid content of unfertilized eggs

Most batches of mature unfertilized eggs contain between 1 and 2.5 mg. of lactic acid per 100 mg. of protein N. In most cases the amount did not change significantly with time after removal of the eggs from the ovary. In very rare cases, values as high as 5 to 10 mg. per 100 mg. of protein N were reached half an hour after shedding, but in such cases the amount diminished in next one or two hours.

Lactic acid production at the time of fertilization

Estimations of lactic acid in the period immediately following

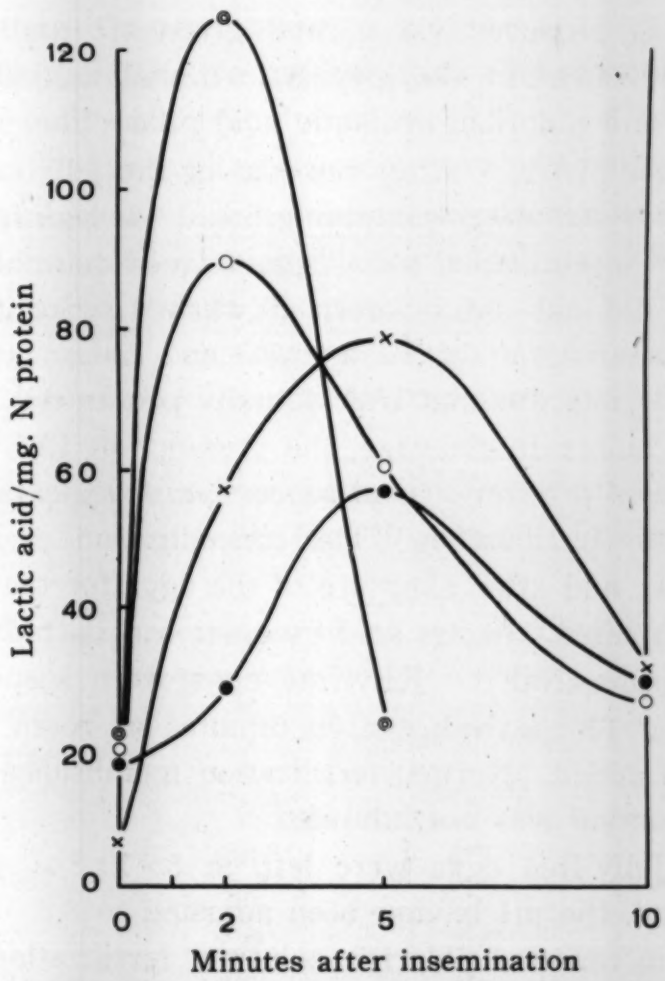


Fig. 1. Transient production of lactic acid after fertilization. *Hemicentrotus pulcherrinus*. Room temperature: 18-20° C. Four cases of the experiments are shown.

insemination indicated that a rapid production of this acid occurred. Maximal values were reached 2 to 5 minutes after insemination, to be followed by an equally rapid decline. Some of the results are shown in Fig. 1.

Effect of glycolytic inhibitors to lactic acid production

According to CLELAND and ROTHSCHILD (1952 a, b), a typical glycolytic pathway is present in the eggs of the sea urchin, *Echinus esculentus*. In the eggs of the sea urchins, *Strongylocentrotus purpuratus* and *Lytechinus pictus* too, YČAS (1954) arrived at a similar conclusion. Unpublished work by the present author on the eggs of *Hemicentrotus* at low oxygen tensions has also given results consistent with this view.

In order to test whether the lactic acid arising during the fertilization reaction is produced via normal glycolytic pathway, a study was made of the effect of the glycolytic inhibitors, iodoacetoamide, sodium fluoride and phlorizin on lactic acid production.

Iodoacetoamide (IAA): After removal of the jelly coat, the eggs were suspended in sea water containing 5×10^{-4} M IAA for 90 minutes. When they were inseminated, such eggs formed normal fertilization membrane, but did not cleave, even if washed twice in normal sea water before insemination (cf. RUNNSTRÖM and KRISZAT, 1952). This may be taken to indicate that IAA actually penetrated into the egg. In spite of later failure in cleavage, the presence of IAA was without effect on the rapid production and successive disappearance of lactic acid which follows fertilization. The concentration of IAA was increased to 10^{-3} M, and after exposure of the eggs for the same period they were inseminated. Lactic acid production occurred as before.

Sodium fluoride (NaF): Jelly-free eggs were suspended in sea water containing 10^{-2} M NaF for 70 minutes at room temperature. Sperm was then added. Normal fertilization membranes formed, and lactic acid production was not inhibited.

Phlorizin: Jelly-free eggs were left in 5×10^{-3} M phlorizin sea water for 2 hours, the pH having been adjusted to 8.2. After the addition of sperm in renewed phlorizin solution* fertilization membranes were elevated irregularly, and the eggs developed normally to early

* High percentage of fertilization could not be gained in the solution in which the eggs were left.

blastula, but they could not hatch and gastrulate. Lactic acid production in question was inhibited.

Quantitative analyses of related compounds

Pyruvic acid: Lactic acid and pyruvic acid are easily interchangeable in animal tissues under the influence of lactic dehydrogenase and diphosphopyridine nucleotide. YČAS (1954) found weak activity of lactic dehydrogenase in eggs of *Strongylocentrotus* and *Lytechinus* using THUNBERG technique. Quantitative study of pyruvic acid during the fertilization reaction is therefore of interest.

Estimations were carried out at intervals of 2, 5, 10 and 15 minutes after insemination.

It was found that the content of this acid is very low both in unfertilized and in newly fertilized eggs, and there was no significant change during and after fertilization.

Glycogen: It is natural to suppose that a quantitative change in this compound would occur simultaneously with the transient production of lactic acid following fertilization. As will be discussed later, ÖRSTRÖM and LINDBERG (1950) observed a considerable decrease of alkali stable and alcohol precipitable carbohydrate, which was considered to be glycogen, within ten minutes after fertilization of eggs of *Paracentrotus lividus.* This was also found by LINDBERG (1943) to occur in the eggs of *Echinocardium cordatum* and *Psammechinus miliaris.*

Glycogen was estimated successively after insemination, but no change was observed within the first 15 minutes (Table 1).

Table 1. Glycogen content during and after fertilization
Hemicentrotus pulcherrimus

Minutes after insemination	Glycogen γ/mg. N		
	Exp. 1	Exp. 2	Exp. 3
0 (Unfertilized)	215	320	285
2	210	310	280
5	210	315	290
10	210	315	280
15	200	310	285

Free reducing sugar: Free reducing sugar is said to be absent in the eggs of many species of sea urchins (PEARLZWEIG and BARRON, 1928; HUTCHENS, KELTCH, KRAHL and CLOWES, 1942 and IMMERS, 1952). Other workers (ÖRSTRÖM and LINDBERG, 1940; LINDBERG, 1943 and CHAMBERS and MENDE, 1953) have described, however, a decrease in this compound after fertilization. In eggs of *Arbacia punctulata*, hexokinase activity was demonstrated (KRAHL, KELTCH, WALTERS and CLOWES, 1954).

In the sea urchins studied here no trace of free reducing sugar could be found even though a large quantity of eggs was used.

Pentose: The major pathway of carbohydrate breakdown was considered for a long time to be glycolysis, which give rise to lactic acid under anaerobic conditions. However, a number of facts have been accumulated which suggest that there is another route of carbohydrate breakdown with the participation of atmospheric oxygen (cf. DICKENS, 1953; COHEN, 1954 and RACKER, 1954 etc.). This route, the hexosemonophosphate oxidative route (HMP oxidative route) as it is termed by DICKENS (1952), involves pentose as intermediates.

In sea urchin eggs, the importance of pentose metabolism was indicated by LINDBERG (1943), and more recently by KRAHL *et al.* (1954, 1955), KRAHL (1956) and GHIRETTI and D'AMELIO (1956).

Accordingly a study was made of the acid soluble pentose content of unfertilized eggs and of eggs during the fertilization reaction.

In no series of estimations could any significant change in pentose content be found (Table 2).

Table 2. Pentose content during and after fertilization
Hemicentrotus pulcherrimus

Minutes after insemination	Pentose γ/mg. N protein		
	Exp. 1	Exp. 2	Exp. 3
0 (Unfertilized)	68.8	41.5	50.0
2	70.0	41.3	50.0
5	68.3	41.3	50.0
10	68.3	41.0	50.0
15	68.3	41.5	50.0

Acid hydrolyzable carbohydrate: According to HUTCHENS *et al.* (1942) eggs of *Arbacia* contain about 110 mg. of acid hydrolyzable carbohydrate per gram of egg protein, and approximately half of this carbohydrate was accounted for as glycogen. In other sea urchins, *Echinus esculentus*, *Echinocardium cordatum*, *Paracentrotus lividus*, *Sphaerechinus granularis* and *Strongylocentrotus droebachiensis*, IMMERS (1952) found carbohydrate compounds, other than glycogen, which contain ribose, glucose, galactose and mannose, and amino sugar as glucosamine.

In the sea urchins studied here a rapid and considerable decrease in the acid hydrolyzable carbohydrate was found to occur after fertilization (Table 3).

Table 3. Acid hydrolyzable carbohydrate content during and after fertilization
Hemicentrotus pulcherrimus

Minutes after insemination	Acid hydrolyzable carbohydrate γ/mg. N		
	Exp. 1	Exp. 2	Exp. 3
0 (Unfertilized)	595	680	500
5	470	—	—
10	470	570	—
15	312	570	325
Decrease	283	110	175

DISCUSSION

A marked increase in the lactic acid content was found to follow fertilization of the eggs of the Japanese sea urchins studied in this paper. This observation conflicts with those of other workers who have been unable to detect any such a change.

This difference may be ascribed either to the use of different species of sea urchins or more probably to the fact that other investigators performed their estimations at longer intervals after insemination than the author did. The transient production and successive consumption of lactic acid found in the present study might have thus been overlooked.

It is of interest that glycolytic inhibitors such as iodoacetoamide

and sodium fluoride do not inhibit the lactic acid production. Eggs of *Hemicentrotus* accumulate an abundance of lactic acid under low oxygen tension, and this accumulation is inhibited by such glycolytic inhibitors (AKETA unpublished). There is, therefore, no reason to suppose that the eggs are impermeable to these inhibitors.

One way out of the dilemma created by the inhibitor studies would be to postulate that the lactic acid produced during the fertilization reaction arises by some pathway other than glycolysis. In this connection it is worthy of note that many workers have suggested the presence of oxidative pathway of carbohydrate in sea urchin eggs (LINDBERG, 1943; LINDBERG and ERNSTER, 1948; KRAHL, KELTCH, WALTERS and CLOWES. 1954, 1955; KRAHL, 1956 and GHIRETTI and D'AMELIO 1956). According to LINDBERG (1943) and LINDBERG and ERNSTER (1948), oxidation is not inhibited by glycolytic inhibitors.

Phlorizin checks phosphorylation of intermediates of carbohydrate degradation (LUNDSGAARD, 1933) and is known to be a strong inhibitors of phosphorylase (*e.g.* BERNFELD, 1951). ROTHSCHILD (1939) found that the production of the unknown acid in cytolyzing eggs of the sea urchin, *Echinus esculentus,* was inhibited by phlorizin. According to ÖRSTRÖM and LINDBERG (1940), glycogen of eggs of *Paracentrotus* did not disappear after fertilization in the presence of phlorizin, and a similar result was obtained by LINDBERG (1943) using eggs of *Psammechinus* and *Echinocardium.* Observed effect of phlorizin to inhibit lactic acid production in the eggs of the Japanese sea urchins used probably indicates that phosphorylation is involved in this process.

RUNNSTRÖM (1933) discovered, using manometric technique, that the eggs of *Paracentrotus* evolved carbon dioxide suddenly after fertilization. This is derived from the bicarbonate present in the surrounding sea water, following the diffusion of an acid out of the egg. Such a phenomenon was also reported by several other workers (BOREI, 1933; ÖRSTRÖM, 1935 and LASER and ROTHSCHILD, 1939).

The nature of this acid is as yet unknown, but it is currently believed to be other than lactic acid, largely because no change in lactic acid following fertilization could be detected analytically, and fluoride and monoiodoacetate did not inhibit acid production.

Although no quantitative comparisons have yet been made in the present materials, it is still premature to deny the possibility of a relation between the manometric production of unknown acid and the

lactic acid production shown in this paper.

The disappearance of glycogen after fertilization was discovered in eggs of *Paracentrotus* by ÖRSTRÖM and LINDBERG (1940), and also in eggs of *Echinocardium* and *Psammechinus* by LINDBERG (1943). These workers analyzed the alkali stable and alcohol precipitable carbohydrate and regarded this as glycogen. According to HUTCHENS *et al.* (1942), this substance corresponds to typical animal glycogen in optical rotation, yield of glucose upon hydrolysis, and reaction with iodine.

In the present study, no degradation of glycogen could be observed, even though the method of LINDBERG (1943) was meticulously followed. This may, of course, be due to differences in the degree of polymerization of the polysaccharide material present in these sea urchins.

Considerable decreases in the acid hydrolyzable carbohydrate of the egg are found in the period of lactic acid production. This carbohydrate fraction includes glycogen, which does not decrease after fertilization. The substrate for the lactic acid produced after fertilization must, therefore, be some oligosaccharide material not precipitable by 60% alcohol. The identification of this compound must await further work.

SUMMARY

1. An abrupt and transient production of lactic acid was observed 2 or 5 minutes after fertilization in eggs of sea urchins, *Hemicentrotus pulcherrimus*, *Heliocidaris classispina* and *Pseudocentrotus depressus*.

2. This production was inhibited by phlorizin but not by sodium fluoride and monoiodoacetoamide.

3. No quantitative change in glycogen, pentose and pyruvate was observed to follow fertilization of the egg, and no trace of free reducing sugar was found.

4. Acid hydrolyzable carbohydrate decreased rapidly after fertilization. The detailed chemical nature of this carbohydrate fraction is as yet unknown.

5. It was suggested that lactic acid was produced through another phosphorylative pathway than glycolysis from acid hydrolyzable carbohydrate.

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ON THE REGIONAL DIFFERENCE IN THE NATURE OF THE CORTEX OF THE SEA URCHIN EGG DURING CLEAVAGE

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When the susceptibility of the cleaving sea urchin egg to various agents was studied in a previous paper (1954 a), a conclusion was reached that the nature of the egg surface differed between the furrow and the polar region. This conclusion, in turn, may suggest that the egg surface or the cortex might play an important role in cleavage. The former half of the present paper concerns an extension of the previous study.

Recently, MOTOMURA (1954) reported a method to isolate the cortex from unfertilized eggs of the sea urchin (*Hemicentrotus pulcherrimus*). Employing MOTOMURA's method with a slight modification, the present author found regional differences in the nature of the cortex of cleaving eggs. Similar differences were also found in the cortex isolated from the parthenogenetic merogones.

MATERIAL AND METHODS

Most of the experiments were carried out using eggs of the sea urchin, *Hemicentrotus pulcherrimus*. Although *Anthocidaris crassispina*, *Temnopleurus toreumaticus* and *Pseudocentrotus depressus* were also used in some experiments, *Hemicentrotus* was found to be the most suitable material.

In the first series of experiments, effects of "sodium isomytal,"* 2, 4-dinitrophenol and sodium azide were studied. A 10 per cent solution of sodium isomytal in distilled water, M/1,000 solution of 2, 4-dinitrophenol in sea water and N/10 solution of sodium azide in distilled water were taken as stock solutions. Graded concentrations

* "Sodium isomytal" (Nippon Shinyaku Co. Ltd.) is a commercial name of sodium 5, 5-isoamyl ethyl barbiturate.

were made up by successive half-dilutions with sea water.

Heat-treatments of eggs were also done in the same procedure as described in the previous paper (1954 a). Fertilized eggs were exposed to 24°, 26° or 28° C. at their various stages. After 30 minutes' treatment, they were returned to room temperature (20° C.) and the effect of warming on the egg surface was examined.

In the isolation of the egg cortex, MOTOMURA's method was adopted with some modification as deceribed below. First, normally fertilized eggs were deprived of both the fertilization membrane and the hyaline layer by repeatedly blowing and sucking with a pipette in an isotonic solution of urea. This procedure was carried out about two minutes after insemination, just after the membrane had been raised and before it had hardened. The reason why the urea solution was used instead of Ca-Mg-free sea water was the convenience for easy removal of the fertilization membrane and the hyaline layer. Eggs with the hyaline layer were unsuitable for the isolation of the cortex, because the layer shrank and became tough in an acid solution employed in subsequent procedure.

The denuded eggs were put back into normal sea water and then exposed to N/200 H_2SO_4 in distilled water at various stages of cleavage. 20 to 40 minutes after this treatment, these eggs were violently blown and sucked with a pipette at least for 5 minutes. The cortex thus isolated was collected by a hand-centrifuge. The cortex of the eggs previously treated with various agents (Monogen, urethane and colchicine etc.) was also isolated in the same way.

In another series of experiments, the unfertilized egg was centrifuged (about 10,000 *g*.) into halves of a light nucleated part and a heavy non-nucleated one. These egg fragments were fertilized or activated parthenogenetically, and their cortex was isolated in the same way during cleavage.

For parthenogenetic merogony, an isotonic solution of urea was used as an activating agent. Both the fertilization membrane and the hyaline layer were not formed when eggs were activated by more than 3 minutes' immersion in the urea solution. At the same time, non-nucleated egg fragments were treated with the urea solution for 3 minutes. After 15 minutes, they were treated with hypertonic sea water (8 cc. of 2.5 N NaCl plus 50 cc. of normal sea water) for 20–40 minutes and put back into normal sea water again.

Experimental Results

I. Difference in susceptibility of the egg surface between the furrow and the polar region of cleaving eggs

A) Experiments with sodium isomytal. When fertilized eggs are immersed in a 5 per cent solution of sodium isomytal (pH 9.2–9.4) just before the initiation of the first cleavage, the equatorial region or the presumptive cleavage furrow, bulges higher than the other portions as shown in Figure 1. No such change occurs in eggs which have already started their cleavage.

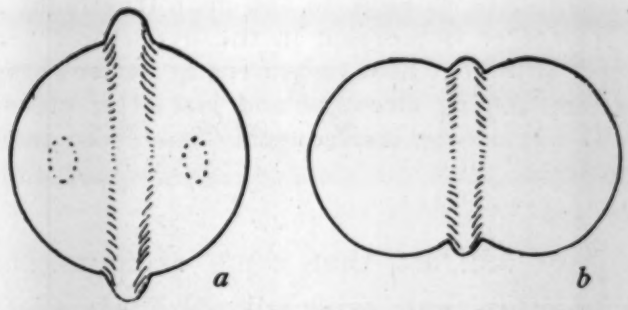


Fig. 1. Transformation of an egg which was treated with isomytal just before the first cleavage.
a: An egg just before cleavage. *b*: A cleaving egg.

B) Heat-treatment. A notable effect of heat is obtained when denuded eggs 5 minutes after fertilization are exposed to 26° C. for 30 minutes. When the eggs are returned to room temperature after the heat-treatment, some eggs cleave normally and some fail to cleave. In the latter, the egg cortex in the equatorial region becomes yellow and thicker. In these eggs cytoplasmic blisters are produced as a band as shown in Figure 2 *a*. These characteristic features gradually fade toward the resting stage. At the second cleavage, the same phenomena are observed in the direction perpendicular to that of the first cleavage by the same procedure.

When heat-treated eggs which failed to cleave, are put into hypertonic sea water (6 cc. of 2.5 N NaCl solution plus 20 cc. of normal sea water), the equatorial region becomes higher than the rest, although the length of the egg diameter in the direction of the spindle axis remains unchanged (Fig. 2 *d*). In the cleaved eggs, after the same procedure, a somewhat different deformation takes place as shown in Figure 2 *e* and *f*.

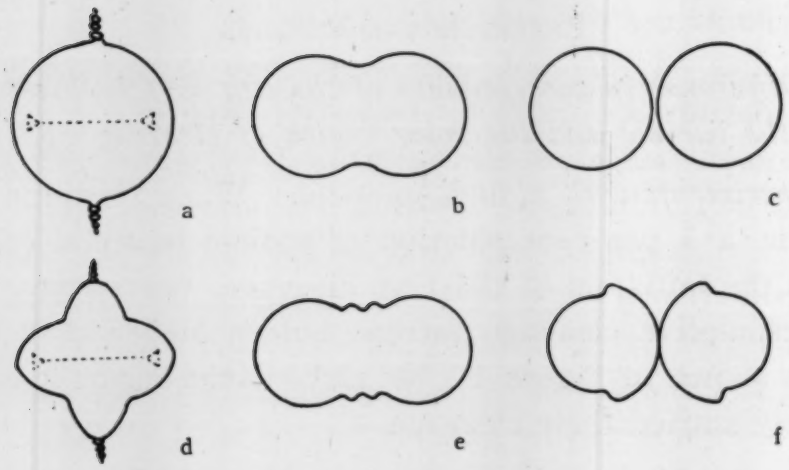


Fig. 2. Response of eggs to the heat treatment and their transformation after immersion in the hypertonic sea water.

a-c: Eggs after the heat treatment at various stages; just before cleavage, during cleavage and just after cleavage.

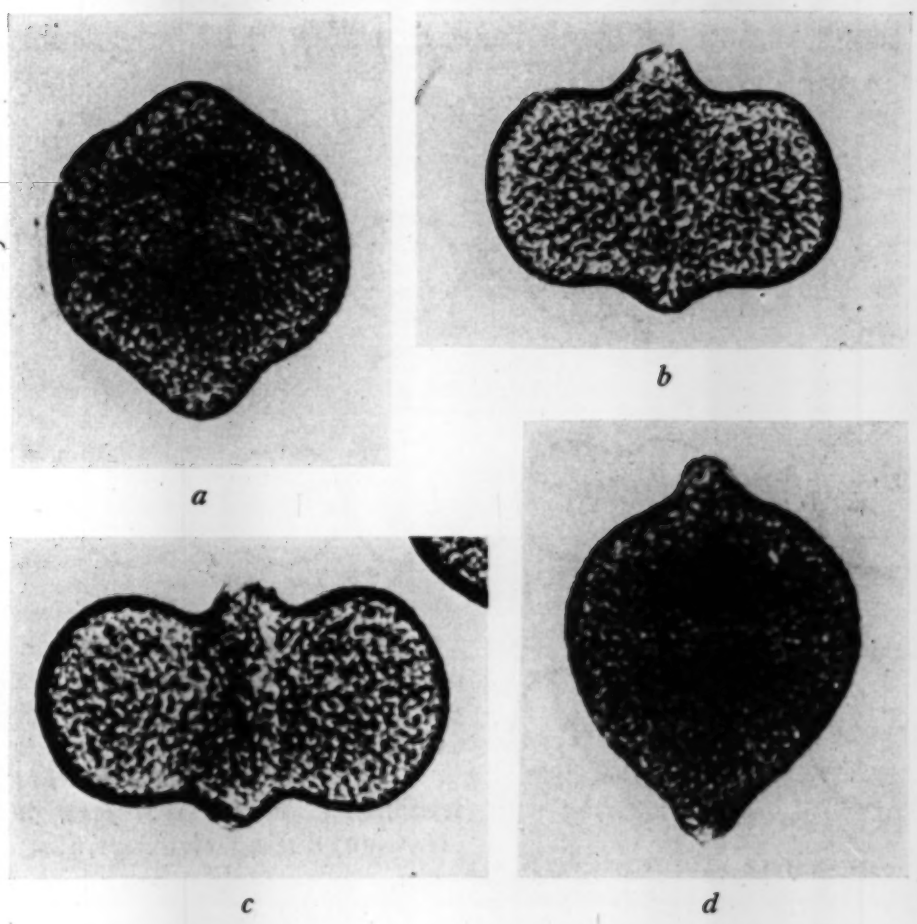
d-f: The same eggs subsequently immersed in the hypertonic sea water.

These facts may indicate that some regional characteristics in the nature of the cytoplasm, especially of the cortex, are made apparent by the heat-treatment. From the shape of the eggs shown in Figure 2, it may also be suggested that these regional differences are concerned with rigidity difference of the cortex and the mitotic spindle.

C) *Experiments with dinitrophenol and sodium azide.* In the next series of experiments, denuded eggs are exposed to various concentrations of 2, 4-dinitrophenol or sodium azide in sea water at 5 or 45 minutes after fertilization to see their responses.

Most significant results are obtained with M/8,000 dinitrophenol and M/80 sodium azide. In these solutions, the eggs undergo various changes in shape as shown in Figure 3, all of which occur only in the region corresponding to the presumptive cleavage furrow. The same is also true with polyspermic eggs. These results are obtained only when the eggs are treated with the reagent before the initiation of cleavage. No change occurs when the treatment is given after cleavage has started.

D) *Experiments with hypotonic sea water, urethane sea water and urea-urethane solution.* Eggs deprived of both the fertilization membrane and the hyaline layer are transferred into a 50 per cent hypo-



a *b* *c* *d*

Fig. 3. Transformation of the furrow region after the treatment with DNP.
a and *d*: Eggs just before cleavage. *b* and *c*: Cleaving eggs.

tonic sea water or a M/2 urethane sea water (5 parts of 1 M urethane plus 5 parts of normal sea water) at various stages from just before the first cleavage up to the beginning of the second one.

Some typical results are represented in Figure 4. When eggs are treated just before the first cleavage, the cortex in the equatorial region changes its color to reddish orange, simultaneously increasing its thickness. The inner cytoplasm in this region gradually becomes transparent appearing as though the eggs have already completed the first cleavage. When the treatment is carried out during or immediately after cleavage, similar changes occur both in the cortex and the cytoplasm in the region of the cleavage furrow or plane, as shown in Figure 4 (*g* and *j*). However, when eggs are put into the solution

about 10 minutes after the completion of the first cleavage, the cortex in the polar region becomes reddish orange, with no accompanying change in the cytoplasm.

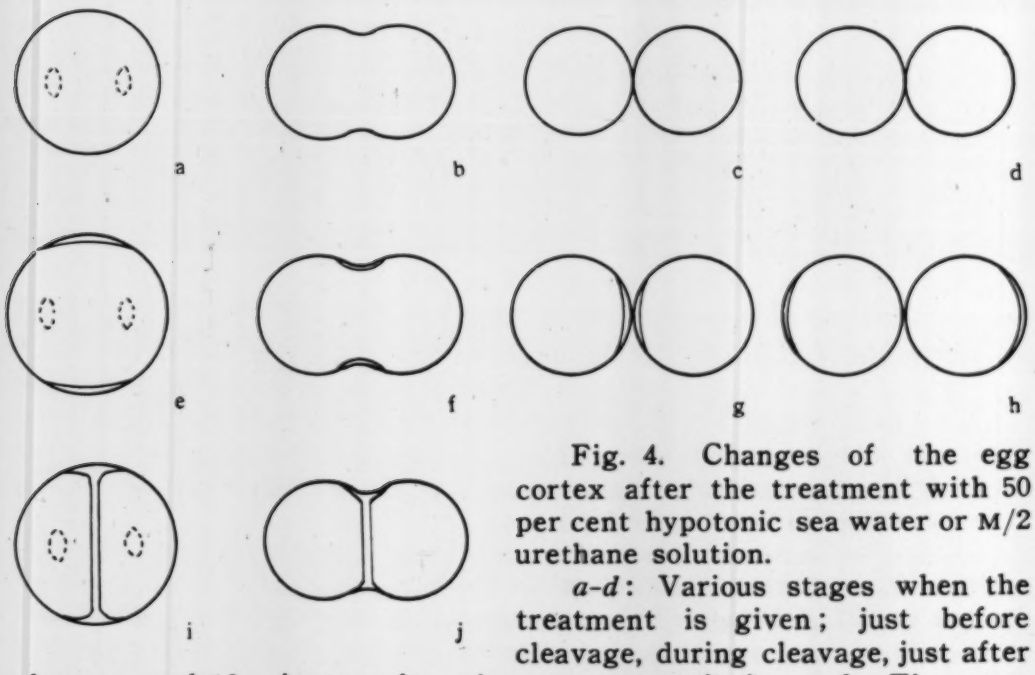


Fig. 4. Changes of the egg cortex after the treatment with 50 per cent hypotonic sea water or M/2 urethane solution.

a-d: Various stages when the treatment is given; just before cleavage, during cleavage, just after cleavage and 10 minutes after cleavage, respectively. *e-h*: The same eggs after the treatment. *i-j*: Further changes of the eggs, the inner cytoplasm at the equatorial region becoming transparent.

Similar results are also obtained in the course of the second cleavage. If eggs are treated at the beginning of the second cleavage, the cortex in the equatorial region of each blastomere becomes reddish orange and the inner cytoplasm in this region becomes transparent.

When denuded eggs before or during the first cleavage, are exposed to a urea-urethane solution (5 parts of 1 M urea solution plus 5 parts of 1 M urethane solution), majority of them cytolyze before long, but the rest show large transparent blisters on the cleavage plane (Fig. 5). On the other hand, when this treatment is carried out about 10 minutes after the completion of the first cleavage, such transparent large blisters appear in the polar region, but not in the cleavage plane. This is also the case in the course of the second cleavage.

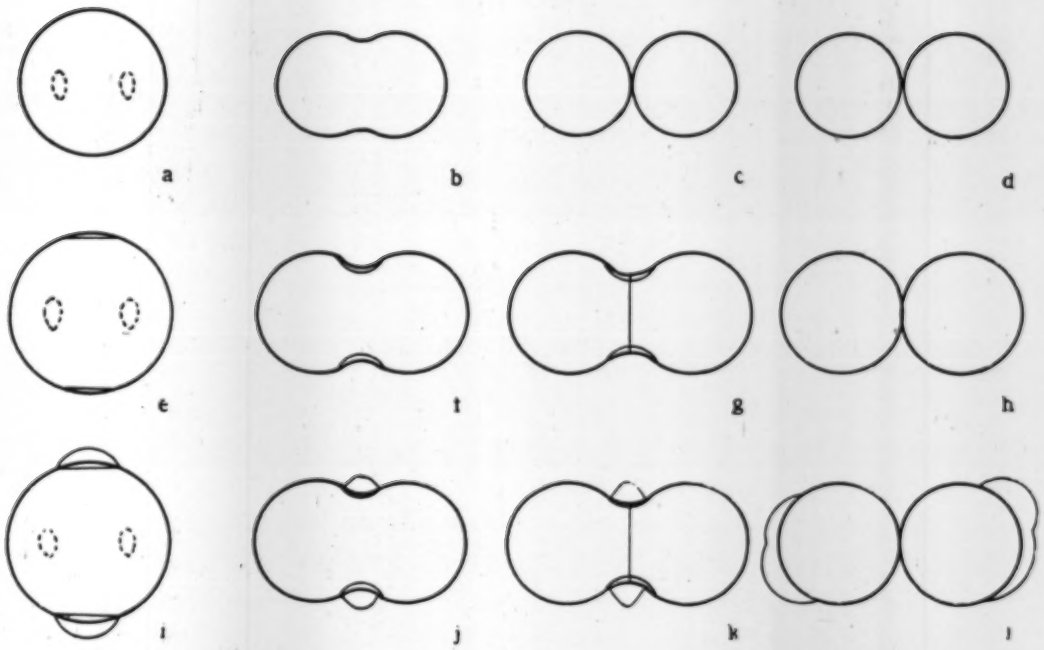


Fig. 5. Changes of the egg cortex after the treatment with urea-urethane solution.

a-d: Various stages of treatment; the same as in Fig. 4 *a-d*. *f-h*: The same eggs after the treatment. *i-l*: Further changes of the eggs, showing blisters in the furrow and the polar region.

II. Regional difference of the cortex isolated from the cleaving egg

A) Normal fertilized eggs. When fertilized eggs just before the onset of the first cleavage are treated with a N/200 H_2SO_4 solution, the furrow cortex becomes thick and blackish, as shown in Figure 6 *a*. This is also the case with cleaving eggs (Fig. 6 *b*, *e* and *f*). If these eggs are violently sucked in and out with a pipette, their cortex can be easily isolated, though somewhat broken (Fig. 6 *c*). Figure 6 *d* is a view perpendicular to Figure 6 *c*, illustrating that the egg cytoplasm has been dispersed.

When eggs are exposed to the acid solution soon after the completion of cleavage, a blackened and X-shaped thickening of the cortex appears in the cleavage plane (Fig. 6 *g*). It is possible to isolate such cortex as an X-shaped fragment as shown in Figure 6 *h*. It is worth noting that such a difference in the cortex is not found if eggs are treated with the acid solution 15 minutes after the completion of cleavage. However, the regional difference of the cortex becomes

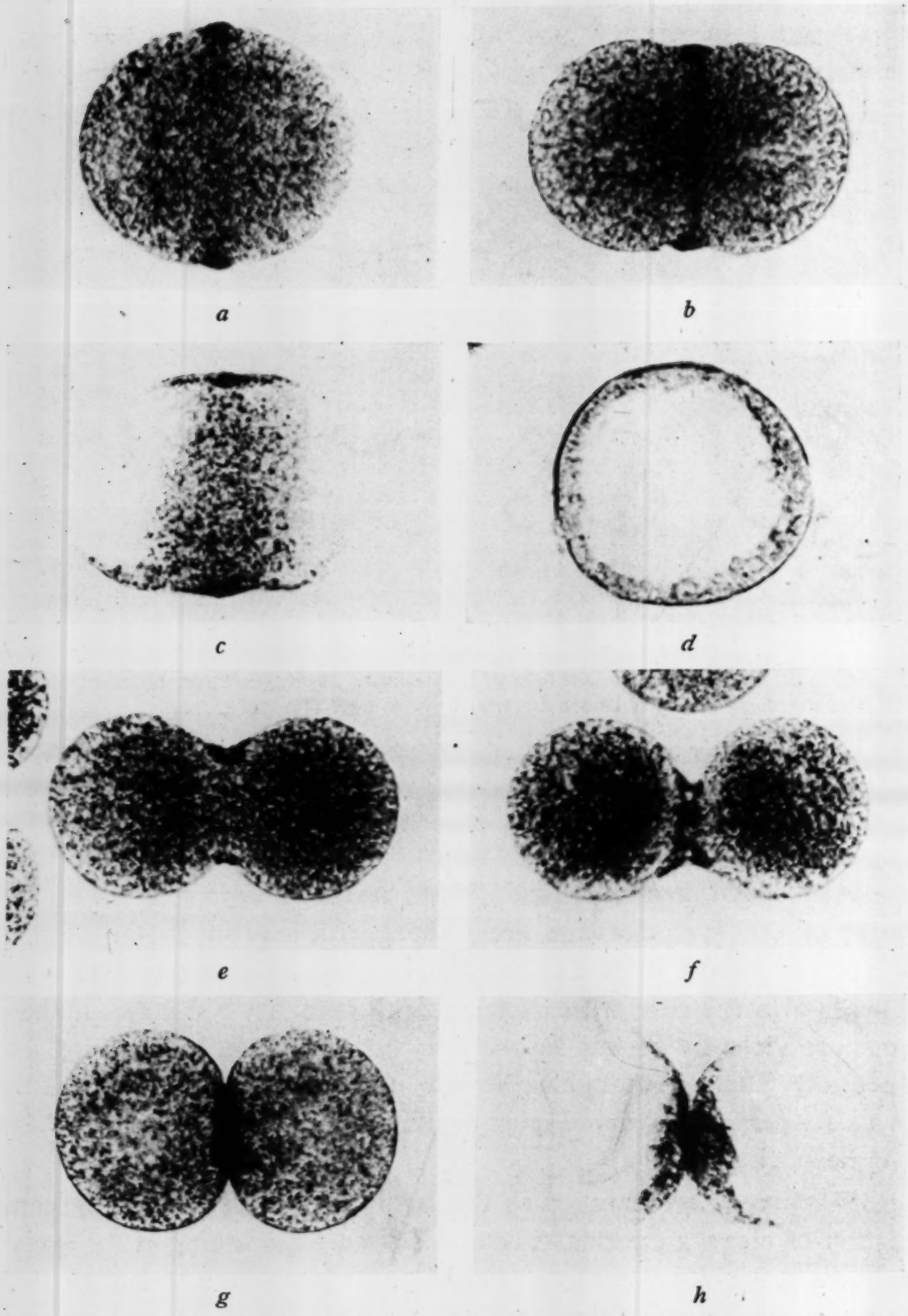


Fig. 6. Regional differences of the cortex in cleaving eggs after application of MOTOMURA's method (1954) for the cortex isolation.

a: An egg just before the cleavage. *b*: A cleaving egg. *c*: A cortex isolated from the egg shown in 6*b*. *d*: A side view of the isolated cortex shown in 6*c*. *e* and *f*: Cleaving eggs at later stages than 6*b*. *g*: A cleaved egg. *h*: An X-shaped cortex isolated from the egg shown in 6*g*.

clear again just before furrowing of the second cleavage, and thus the eggs are repeating the same change in the cortex as that in the course of the first cleavage.

In addition, if polyspermic eggs are treated with the acid solution, the furrow cortex becomes thick and blackish as shown in Figure 7.

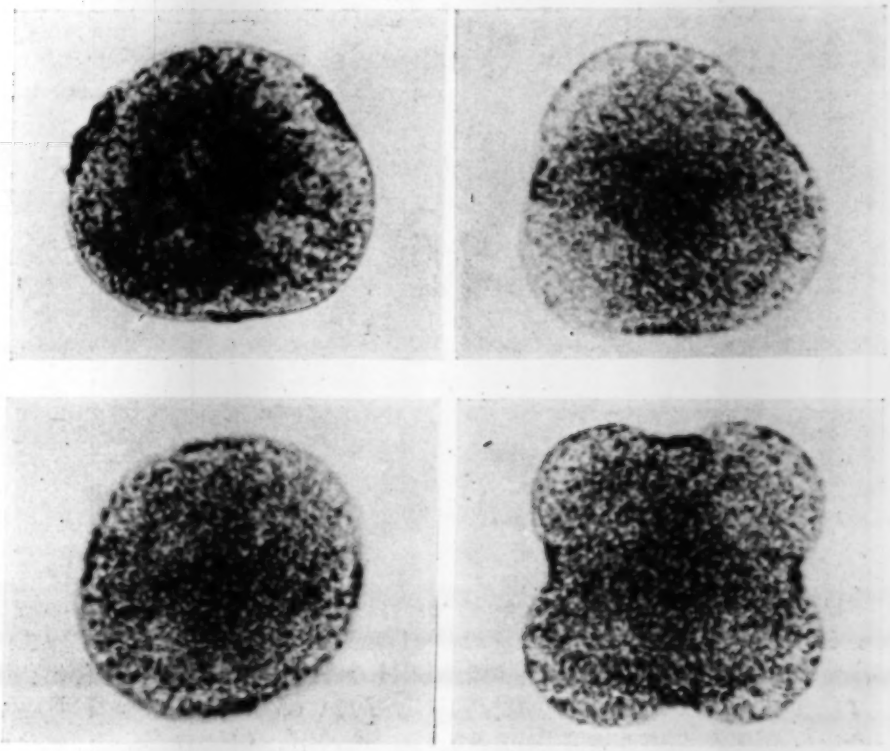
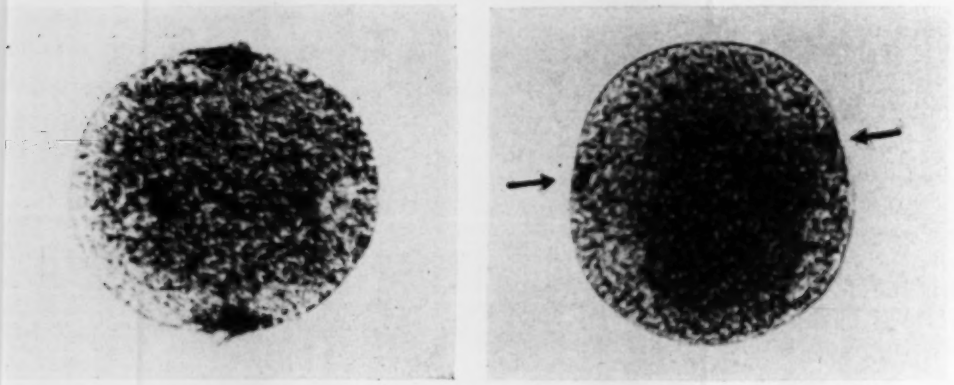


Fig. 7. Regional differences of the cortex in polyspermic eggs just before or during cleavage. (After application of MOTOMURA's method for the cortex isolation.)

B) Urethane-treated and colchicine-treated eggs. It was reported in a previous paper (KUNO, 1954 b) that when fertilized eggs are immersed in a urethane solution of an appropriate concentration, the cytoplasmic division is suppressed while the nuclear division goes on normally, so that multinucleated eggs are formed. When eggs just before or during the first cleavage are put into the urethane sea water (M/16 or M/8), the first cleavage occurs but subsequent cytoplasmic divisions are suppressed. The cortex was isolated from such urethane-treated eggs. It was found that the cortex isolated from the furrow region or the cleavage plane shows black thickening quite similar to that from control eggs.

When eggs are put into a M/16 urethane sea water 5 minutes

after fertilization, the mitotic spindle is formed normally but the aster does not develop. Isolation of the cortex is performed when their spindle has fully elongated. As shown in Figure 8 *a*, a black thickening of the cortex appears in the equatorial region of the egg. However, such a change is not so clear when isolation is performed at a later stage when control eggs have completed their first cleavage. After a full development of the second mitotic spindle, the regional changes of the cortex becomes visible again as shown in Figure 8 *b*.



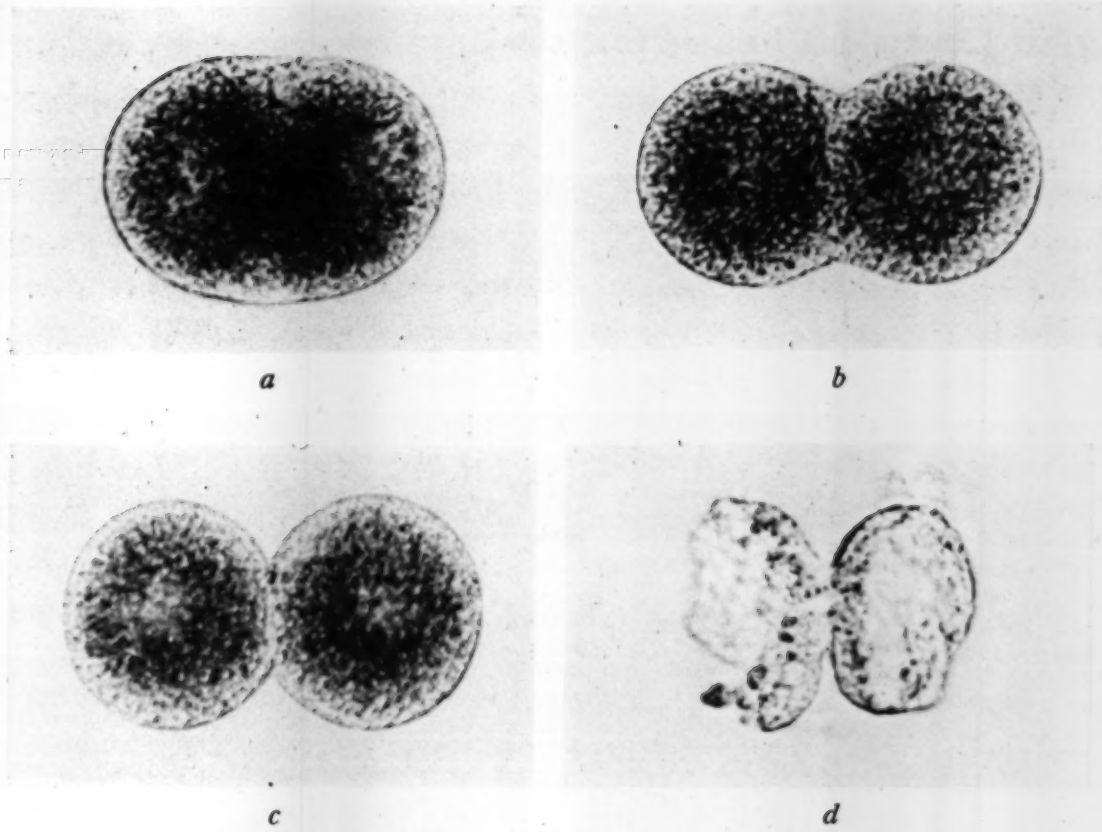
a *b*

Fig. 8. Regional differences of the cortex in urethane-treated eggs in which the cytoplasmic division is suppressed. (After application of MOTOMURA's method for the cortex isolation.)
a: A stage corresponding to the first cleavage.
b: A stage corresponding to the second cleavage.
Note that the changes of the cortex are found in the region perpendicular to that of the first. Further explanation in text.

When urethane sea water of a higher concentation is used, neither spindle formation nor nuclear division takes place and no regional difference of the cortex can be found.

Similar experiments were performed with colchicine with similar results to those of urethane.

C) Monogen-treated eggs. It was found in the previous work (KUNO, 1954 b) that Monogen, a detergent consisting chiefly of a mixture of myristyl sulphate and lauryl sulphate, suppresses the cytoplasmic division of sea urchin eggs without affecting the nuclear division. Cleaving eggs are immersed in 1/64 per cent Monogen sea water for 5 minutes, and then treated with a N/200 H_2SO_4 solution. They do not show regional changes in the cortex (Fig. 9).



a *b*

c *d*

Fig. 9. Regional differences of the cortex in Monogen-treated eggs after application of MOTOMURA's method for the cortex isolation.
a-c: Monogen-treated eggs at different stages. *d*: A cortex isolated from the egg shown in 9*c*. Note that no regional difference is formed.

In the next experiments, fertilized eggs are exposed to a rather strong solution of Monogen for 5 minutes at various stages before the first cleavage and then returned to normal sea water. These eggs do not show cleavage. No regional changes in the cortex are found after the acid treatment even at the stage which corresponds to cleavage in control eggs. This result differs markedly from that of the urethane treatment described in the previous section.

A result essentially similar to Monogen is also obtained by Duponol treatments.

D) Parthenogenetically activated eggs, enucleated or nucleated. By menas of the same procedure, a similar cortical differentiation is found in the furrow region of eggs developed parthenogenetically, whether in normal bipolar cleavage or whether in abnormal multipolar cleavage (Fig. 10*a*). It was further found that such a cortical

differentiation takes place even in the enucleated halves obtained by centrifuging and induced to develop parthenogenetically, as shown in Figure 10 *b*, *c* and *d*.

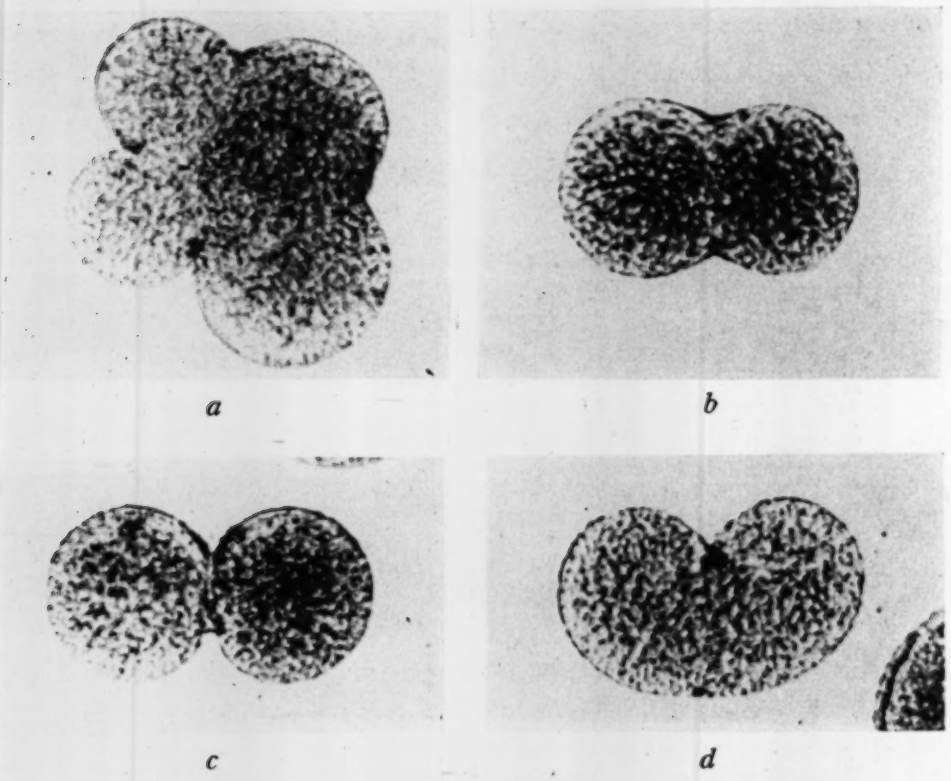


Fig. 10. Regional differences of the cortex in parthenogenetic eggs and merogones. (After application of MOTOMURA's method for the cortex isolation.)
a: Abnormal cleavage of an artificially activated egg.
b and *c*: Cleavage of parthenogenetic merogones.
d: Abnormal cleavage of a parthenogenetic merogones, showing the regional differences of the cortex on one side.

DISCUSSION

The results described in the first half of this paper ascertain the previous conclusion that the nature of the native egg surface differs between the furrow and the polar region. The regional differences of the cortex become alternately evident and concealed in accordance with the cleavage cycle. It was also confirmed that the surface of the equatorial region is already differentiated at a stage just before cleavage.

The peculiar deformation of eggs after treatment with heat and hypertonicity seems to suggest that the cortex of the future furrow

has become more rigid than the rest in preparation for cleavage. Although this be an artifact, this phenomenon may indicate that the cortex of the equatorial region must have been modified so as to become rigid by the heat-treatment.

When eggs just before cleavage are treated with M/2 urethane sea water or 50 per cent hypotonic sea water, beside the color change and thickening of the cortex in the equatorial region, the inner cytoplasm of this region becomes transparent. This fact indicates that both the cortex and the cytoplasm of this region are affected from before cleavage. This is interesting in connection with MOTOMURA's observation (1950) that at the telophase the basophilic vacuoles gather around the spindle region so that the cytoplasm of the equatorial region becomes porous and at the time of cleavage these vacuoles contribute to the formation of the new cell surface.

Recently, MITCHISON (1952) and SWANN (1952) have put forward the "expanding membrane theory" of cell division. They state that the structural agent is released from chromosomes in the anaphase. After the release this agent reaches by diffusion first the poles of the egg where it makes the cortex to expand. This expansion gradually spreads out from each pole towards the equator, as the agent reaches the subpolar regions. This may mean that the cortex changes chronologically under the influence of the structural agent. Although their theory seems to be quite in harmony with the results described in the present paper, the results of the acid treatment show that differences in the nature of the cortex between the furrow and the polar regions are also found in parthenogenetic merogones which contain no nucleus. Therefore, it appears that at least in such merogones the regional difference is not due to a nuclear influence but to some cytoplasmic changes.

DAN (1954) reported on the cortical movement of dividing *Arbacia* eggs. The cortex exhibits oscillating movement between the furrow and pole in pace with the division cycle. The appearance or disappearance and further reappearance of the regional difference in the cortical nature here reported resemble the cortical movement observed by DAN in their cyclic feature. But much more experiments are needed before the relation between the two can be discussed.

Eggs treated with a detergent such as Monogen or Duponol show neither cleavage nor the regional difference in the cortex. On the

other hand, eggs treated with urethane show the cortical differentiation as normal as controls, even when eggs are exposed to a sufficient concentration to make them multinucleated. These findings fall in line with the previous conclusion (1954 b) that Monogen mainly acts on the egg cortex suppressing the cleavage while urethane is more effective on the inner cytoplasm so that the aster formation is inhibited. It may be considered that the cytoplasmic division fails to take place, either when differentiation of the cortex is arrested or when the aster formation is suppressed.

When eggs have reached a certain stage of mitosis, cytoplasmic division ensues even if the aster is destroyed by colchicine (BEAMS and EVANS, 1940; SWANN and MITCHISON, 1953; KUNO, 1954 b), and urethane (KUNO, 1954 b), or removed by a micropipette (HIRAMOTO, 1956). This suggests that at the beginning the cortex is influenced by the inner protoplasm to acquire regional differences but once the cortex acquires the differentiation, it is capable of furrowing without the aster. It should be noted here that detergents such as Monogen which mainly attack the cortex and cancel the regional differences, can prevent the furrowing even at the final stage.

Finally there remains one more question. When eggs are put into urethane solution 5 minutes after fertilization, they fail to cleave. Notwithstanding, the regionality of the cortex appears as usual. However, if eggs are exposed to urethane immediately before cleavage, no disturbance occurs in the cleavage with the cortical differentiation. Although the cortical differentiation appears distinctly in both cases, in one case cleavage occurs while in the other no cleavage. Another factor besides the visible regional differentiation of the cortex might be needed for the mechanism of furrowing. Since no data has yet been obtained for this factor, discussion on this point must await further researches.

The author wishes to express his sincere gratitude to Prof. K. DAN for his kind criticism and correction of the manuscript.

SUMMARY

1. The eggs of the sea urchin, *Hemicentrotus pulcherrimus* were mostly used.

2. Fertilized eggs were treated with the following agents; iso-

mytal, 2, 4-dinitrophenol, sodium azide, heat, heat-hypertonicity, 50 per cent hypotonic sea water, urethane and urea-urethane. Regional differences in reactions were observed between the furrow and the polar region of the egg during cleavage.

3. The regional difference was perceived distinctly in the cortex isolated from cleaving eggs employing MOTOMURA's method (1954).

4. The regional difference was also found in urethane-treated eggs, in which the cytoplasmic division was inhibited without the suppresion of the nuclear division.

5. The appearance of the regional difference was not affected by colchicine-treatment.

6. Monogen and Duponol showed a decisive effect to prevent the appearance of such a difference.

7. The regional difference of the cortex was revealed even in the parthenogenetic merogones which lacked nuclei.

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CARBOHYDRATE METABOLISM IN DIAPAUSE EGG OF THE SILKWORM, *BOMBYX MORI*

I. DIAPAUSE AND THE CHANGE OF GLYCOGEN CONTENT

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INTRODUCTION

The knowledge on the hormonal control of embryonic diapause of *Bombyx* silkworm has been greatly extended by FUKUDA (1951, 1952) and HASEGAWA (1955). According to them, diapause is induced by a hormone released from the maternal suboesophageal ganglion, the liberation of which is, in turn, controlled by the brain through the nerve cord. When the eggs are exposed to the influence of this hormone, they are of diapause type; in its absence only non-diapause eggs are laid. The term 'diapause factor' has been suggested for this hormone by FUKUDA.

In contrast to the clear-cut evidences existing for the humoral mechanism, little is known concerning the precise role of the diapause factor in the chain of events leading to the onset or termination of diapause. Probably by the presence of the diapause factor, some physico-chemical condition is induced within the eggs, which persists until diapause is broken by low temperature or hydrochloric acid treatment.

The present study is undertaken to analyse this physico-chemical state. For this purpose, the following two points should be investigated in the first place. One is to examine whether or not there is a difference in metabolism between diapause and non-diapause eggs, and the other is to try to find metabolic change, which is closely connected with the onset or termination of diapause. NAKATA (1952) has reported that glycogen in the diapause egg of *Bombyx* decreases

rapidly immediately following the oviposition and it increases again in the later period of diapause.

The present author has studied in detail carbohydrate metabolism in the developing and diapausing eggs of *Bombyx*, and he has found that there is an intimate interrelation between the change in glycogen content and diapause.

Materials and Methods

The materials used were the eggs obtained from the cross between Chinese and Japanese bivoltine races.

As is well known, the type of the egg of this cross is determined by the conditions of temperature and light which prevailed during embryonic development of the maternal generation. Mother moths which were incubated during the egg stage at 25° C. under illumination lay only 'diapause' eggs, and those which were incubated at 15° C. in the dark lay only 'non-diapause' eggs.

Quantitative determination of carbohydrate

As will be shown later by paper chromatography, carbohydrates found in detectable amount within the eggs are confined to glycogen and glucose throughout diapause and embryogenesis. They were determined by the following procedures based upon the colorimetric method of Mendel *et al.* (1954) and Kemp *et al.* (1954).

1) Glucose. 100 eggs* of a known age were ground in 5 ml. of 80% methanol and centrifuged. Glucose is found in the supernatant and glycogen is included within the precipitate. 10 mg. of charcoal was added to the supernatant, and alcohol was removed completely by evaporation under reduced pressure on warm water. 'Deproteinizing solution' (5% trichloroacetic acid containing 0.1% Ag_2SO_4) was added to the remaining aqueous solution to bring the total volume to 5 ml., the suspension being centrifuged. One volume of the supernatant was mixed with 3 volumes of 96% H_2SO_4 and the mixture was heated for exactly 8 minutes in boiling water. The intensity of bluish pink color produced was measured at 520 mμ in a Leitz photometer.

* The total weight of 100 eggs was 56.0 mg. for 'diapause' egg and 54.0 mg. for 'non-diapause' egg at the oviposition time.

2) Glycogen. Since glycogen is insoluble in 80% methanol, it can be extracted from the precipitate after extraction of glucose with methanol. It was resuspended in 10 ml. of deproteinizing solution and heated at 100° C. for 15 minutes. After centrifuging, glycogen in supernatant was determined by the same procedure as that described above for glucose. The quantity of glycogen was represented in terms of glucose.

3) Total carbohydrate. 100 eggs were ground in 10 ml. of deproteinizing solution from the beginning avoiding the use of methanol. The whole suspension was heated at 100° C. for 15 minutes, and then centrifuged. The supernatant was used for color reaction as described above. In all cases, the sum of glucose and glycogen was approximately equal to the amount of total carbohydrate.

Qualitative analysis of carbohydrates with paper chromatography

200 to 500 mg. of eggs were ground in 10 ml. of 80% methanol. After centrifuging, the supernatant was dried completely under reduced pressure. The residue was extracted with a small volume of anhydrous pyridine. A clear solution obtained after centrifuging was employed as a sample for paper chromatography.

As an alternative procedure, the eggs (200 to 500 mg.) were ground in 10 ml. of 5% trichloroacetic acid and centrifuged. The supernatant was washed five times with ether to remove trichloroacetic acid and was dried completely under reduced pressure. The dried residue was divided into two portions, the one being extracted directly with pyridine anhydrous and the other being dissolved in N HCl and hydrolysed at 100° C. for 2.5 hours. The hydrolysate was dried completely after neutralization and was also extracted with pyridine anhydrous. These pyridine extracts were used as samples for paper chromatography. The mixture of butanol, acetic acid and water (4 : 1 : 2) was used as a solvent of development. Aniline hydrogenphthalate sensitive for aldose and orcinol sensitive for ketose were both employed for detection of sugar spots.

Quantitative determination of lactic acid

The micro-method of Hullin *et al.* (1953) was used for determination of lactic acid.

100 eggs were ground in 10 ml. of 5% trichloroacetic acid and

then centrifuged. An aliquot of the supernatant was treated three times with copper-lime ($CuSO_4$ and $Ca(OH)_2$ mixture). 6 volumes of concentrated H_2SO_4 were mixed with one volume of the treated solution on adding $CuSO_4$ solution. The mixture was heated for 30 minutes in water bath at 60° C. and then cooled to 10–15° C. Thereafter *p*-hydroxydiphenyl reagent was added and the mixture was incubated for 20 minutes at 28–30° C. followed by 90 seconds in boiling water. The intensity of violet color produced was measured by Leitz photometer at 550 mμ.

Measurement of oxygen uptake of egg

Oxygen uptake of the eggs was measured by Warburg's manometer at 25° C., using 2 g. of egg.

Results

Changes in content of glycogen and glucose in developing and diapausing eggs

1) '*Non-diapause*' *egg* Total period for embryonic development was about 8.5 days at 25° C.

The variations in the content of glycogen and glucose with respect to the development are shown in Fig. 1. The glycogen content decreased from 1.60 mg. per 100 eggs at the oviposition time to 0.17 mg. at the hatching time. The decrease was relatively slow during the period before blastokinesis stage, which occurred at about half way of development, but the decrease was very rapid during the later half developmental period. The decrease of glycogen is considered to be due to its consumption as an energy source for development.

Although glucose was not detectable in the egg at the oviposition stage, it appeared soon and increased gradually up to 2 days before the hatching time. From this point on, it decreased rapidly until the egg hatched. The maximum value amounted to 0.20 mg. per 100 eggs.

2) '*Diapause*' *egg* The eggs destined to diapause were kept at 25° C. for 30 days after oviposition, and then at room temperature for 160 days (from early in August to early in January). Thereafter

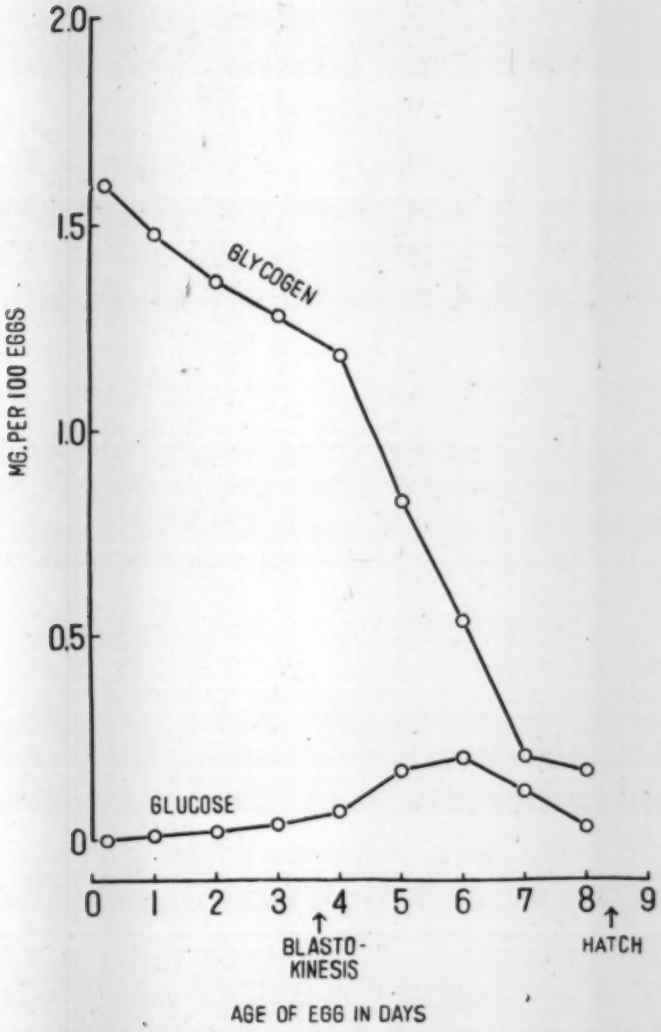


Fig. 1. Changes in content of glycogen and glucose of 'non-diapause' egg during embryogenesis. Glycogen is represented as glucose equivalent.

they were maintained in a refrigerator at 5° C. for 80 days and were put back to 25° C.

The changes of glycogen and glucose of these eggs are indicated in Fig. 2. The amount of glycogen at the oviposition time was 2.21 mg. per 100 eggs, about 35% higher than that found in 'non-diapause' egg. Glycogen decreased gradually during the first day after oviposition, after which it decreased very abruptly. The beginning of the marked decline of glycogen coincided closely with the time when oxygen uptake of the egg also began to fall. This is generally regarded as the initiation of diapause. The content of

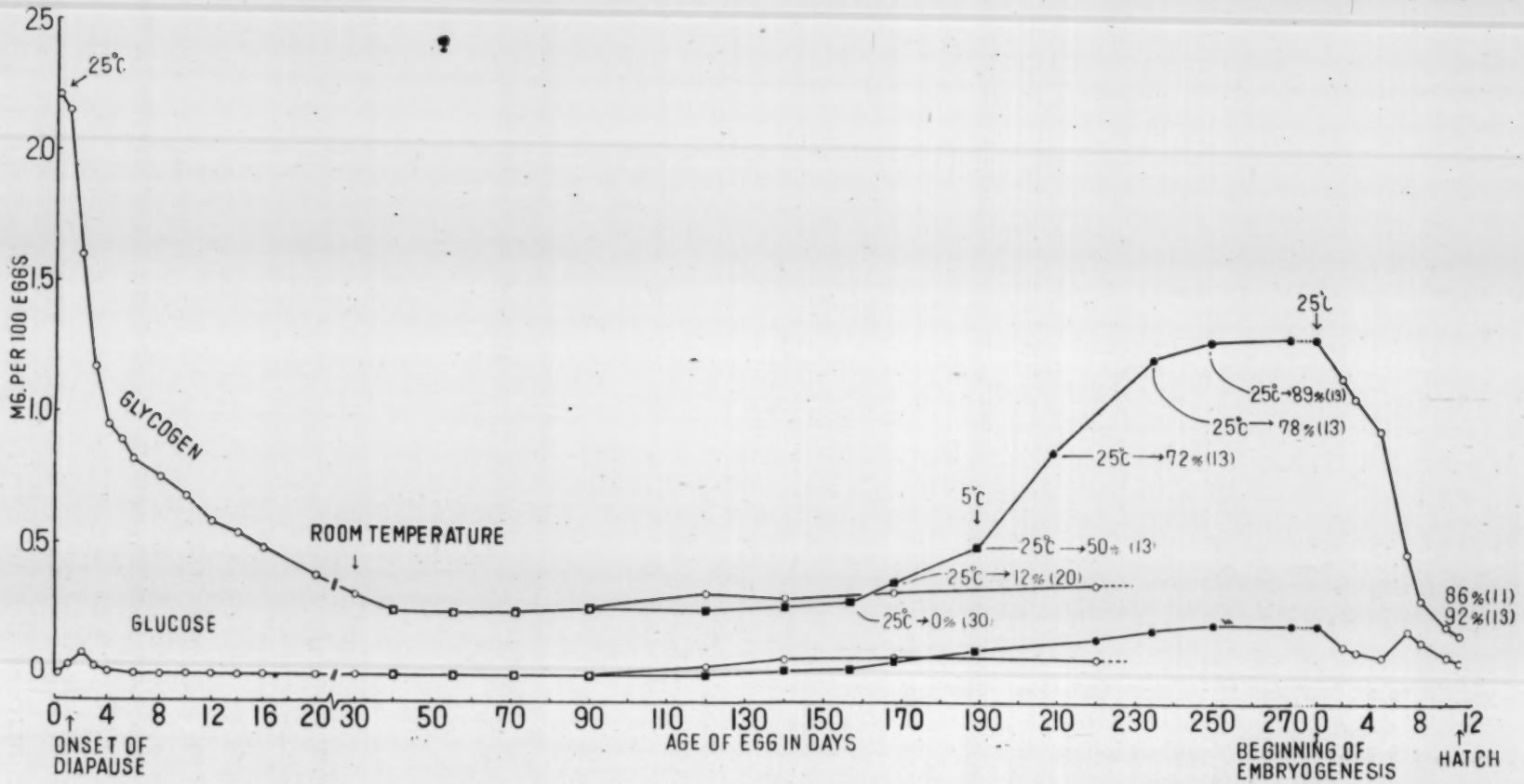


Fig. 2. Changes in content of glycogen and glucose of 'diapause' egg at room temperature during pre-diapause, diapause and post-diapause period. ○: eggs at 25° C., ■: eggs at room temperature, ●: eggs at 5° C. Figures in parenthesis indicate number of days of incubation at 25° C. Figures in percentage represent hatchability.

glycogen at 24 hours after oviposition will hereafter be referred to as 'initial level' or 'initial amount' of glycogen.

The glycogen content decreased very rapidly at first but then more slowly and reached the minimum level (0.25 to 0.30 mg.) at about 30 days after oviposition. Thereafter it changed little until it began to increase at about 160 days after oviposition (early in December). By the time the eggs were transferred to 5° C. at 190 days, a little further increase of glycogen was noticed. The increase continued progressively even at 5° C., and glycogen reached the maximum value, amounting to 1.31 mg. per 100 eggs at about 250 days after oviposition, that is, at about 60 days after the transfer to 5° C. When the eggs, in which glycogen reached the maximum value, were incubated at 25° C., they promptly resumed embryogenesis and glycogen decreased progressively with development as was observed in 'non-diapause' egg. The hatchability of these eggs attained to more than 90% at 11–13 days of incubation at 25° C. On the other hand, the eggs which were incubated while glycogen was still at the lowest level, never hatched out even after 30 days of incubation at 25° C. (see Fig. 2). The hatchability increased seemingly in proportion to the glycogen level, indicating an intimate relationship between the termination of diapause and the recovery of glycogen.

From the above observations it appears that glycogen declines abruptly when the eggs have entered diapause and it nearly recovers the 'initial amount' when diapause is over. This view finds further supports in the following facts. First, when the 'diapause' eggs were converted to non-diapause type (so-called 'artificial non-diapause' egg) by treating with warm HCl (specific gravity 1.075) at 20 hours after oviposition, the variation of glycogen within these eggs was essentially similar to that found in 'non-diapause' egg (Fig. 3). Second, if the 'diapause' eggs weré permanently kept at 25° C., though glycogen decreased in the beginning, it never increased even after 200 days or more after oviposition, except a slight increase toward the end. In this case, 15 to 20% of embryos died in eggs and only 5 to 7% hatched.

Now let us consider the behavior of glucose of the 'diapause' egg. No glucose was found in the egg at the oviposition time. It could be detected in a small quantity soon after glycogen had begun

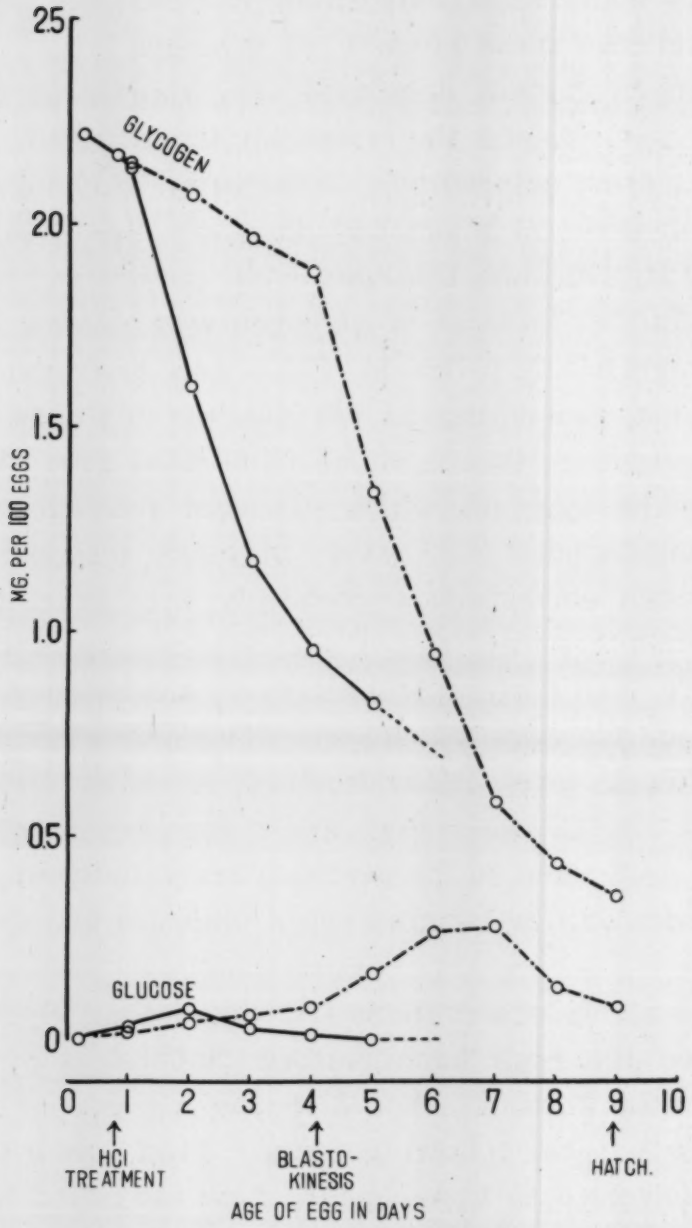


Fig. 3. Changes in content of glycogen and glucose of 'artificial non-diapause' egg during embryogenesis (—·—). ——: the changes when the eggs were kept at 25° C. without HCl treatment.

to show a sharp decrease, but it soon disappeared and no detectable amount was found until glycogen began to increase at about 160 days after oviposition. Then glucose rose gradually, in paralell with the rise of glycogen, up to the maximum value (0.18 mg. per 100 eggs). Thus, the total carbohydrate (glycogen plus glucose) at the

maximum stage attained to about 70% of the 'initial amount' of glycogen.

Further analysis of the relation between diapause and the shift of glycogen level

In the previous section an intimate relationship between diapause and the change of glycogen content has been shown. As a further analysis of this relation, the following experiments have been carried out.

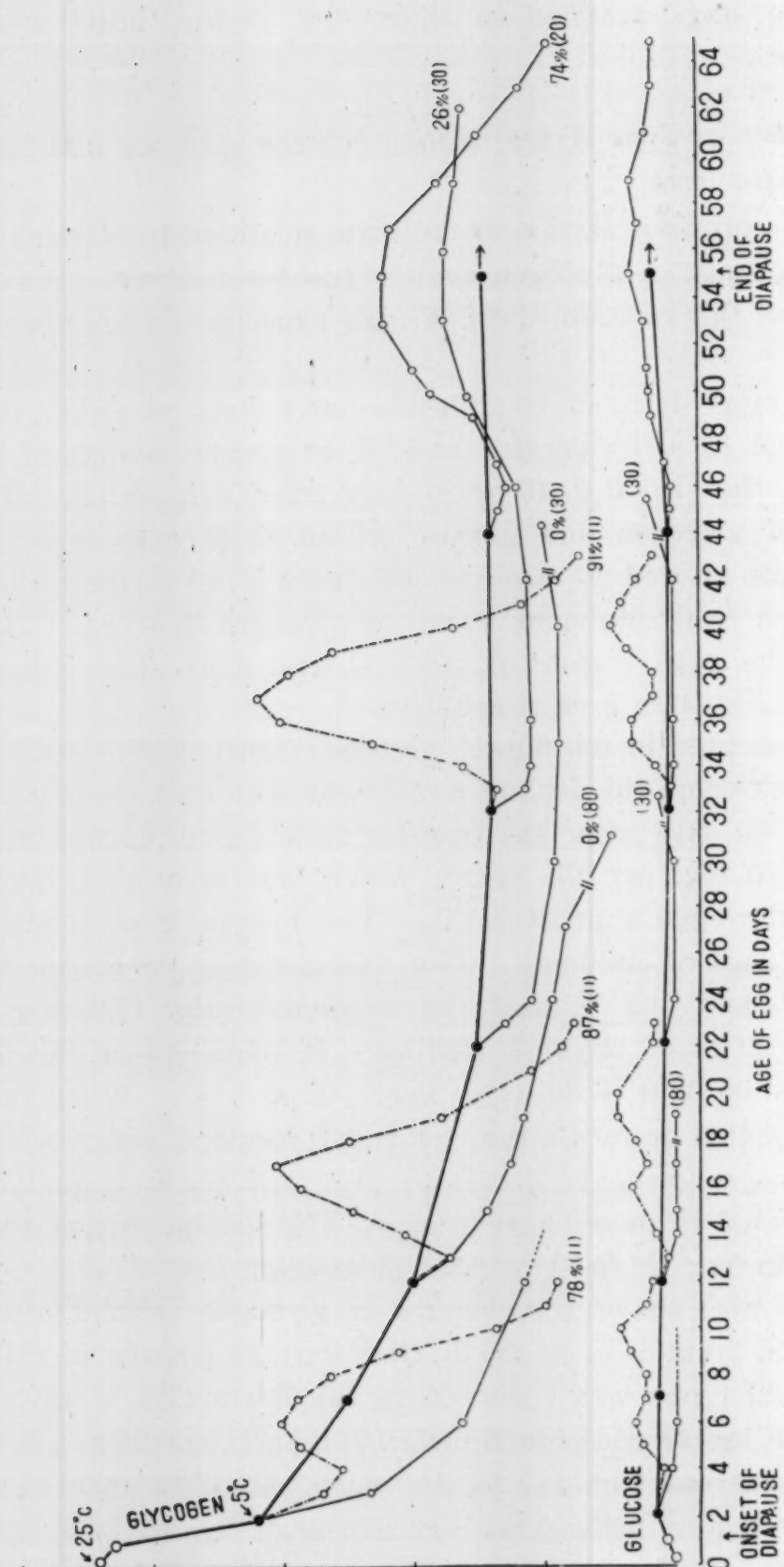
The eggs destined to diapause were kept at 25° C., and then transferred to a refrigerator at 5° C. at various stages of glycogen decrease, that is, at 2, 10 or 20 days after oviposition. The later change of glycogen and glucose within these eggs were followed. The results showed no essential difference in all of these three cases except the final minimum levels of glycogen reached. Hence the data in the eggs transferred to 5° C. at 2 days after oviposition is shown in Fig. 4 as a representative.

As indicated in the figure, glycogen continued to decrease even at 5° C., although the decline was slower than that observed at 25° C. At about 30 days after the transfer to 5 °C., it reached the lowest level* (0.70 mg. per 100 eggs), which was somewhat higher than that of the eggs kept at 25° C. The lowest level persisted until about 50 days of chilling. From this point on, glycogen began to increase again and reached the maximum value (1.63 mg. per 100 eggs) at 90 to 95 days of chilling. It remained at this level for more than 30 days if the eggs were left at 5° C. When these eggs were incubated at 25° C., they promplty resumed embryogenesis and their glycogen contents decreased progressively in a similar way to that observed in 'non-diapause' egg. The hatchability of these eggs attained to 98% at 10–11 days of incubation.

Fig. 4 also shows the changes in glycogen content of the eggs, transferred from 5° C. to 25° C. after various lengths of chilling. A distinct difference was observed in the fluctuation of glycogen according to the time of the transfer. 1) Early transfer. If the eggs were transferred to 25° C. in the early part (less than 30 days) of

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* The minimum levels of the eggs transferred to 5° C. at 10 and 20 days after oviposition were 0.43 and 0.33 mg. per 100 eggs, respectively.


MG. PER 100 EGGS
GLYCOGEN
GLUCOSE
25°C
5°C
26%(30)
74%(20)
0%(30)
91%(11)
0%(80)
87%(11)
78%(11)
(30)
(80)
ONSET OF DIAPAUSE
END OF DIAPAUSE
AGE OF EGG IN DAYS

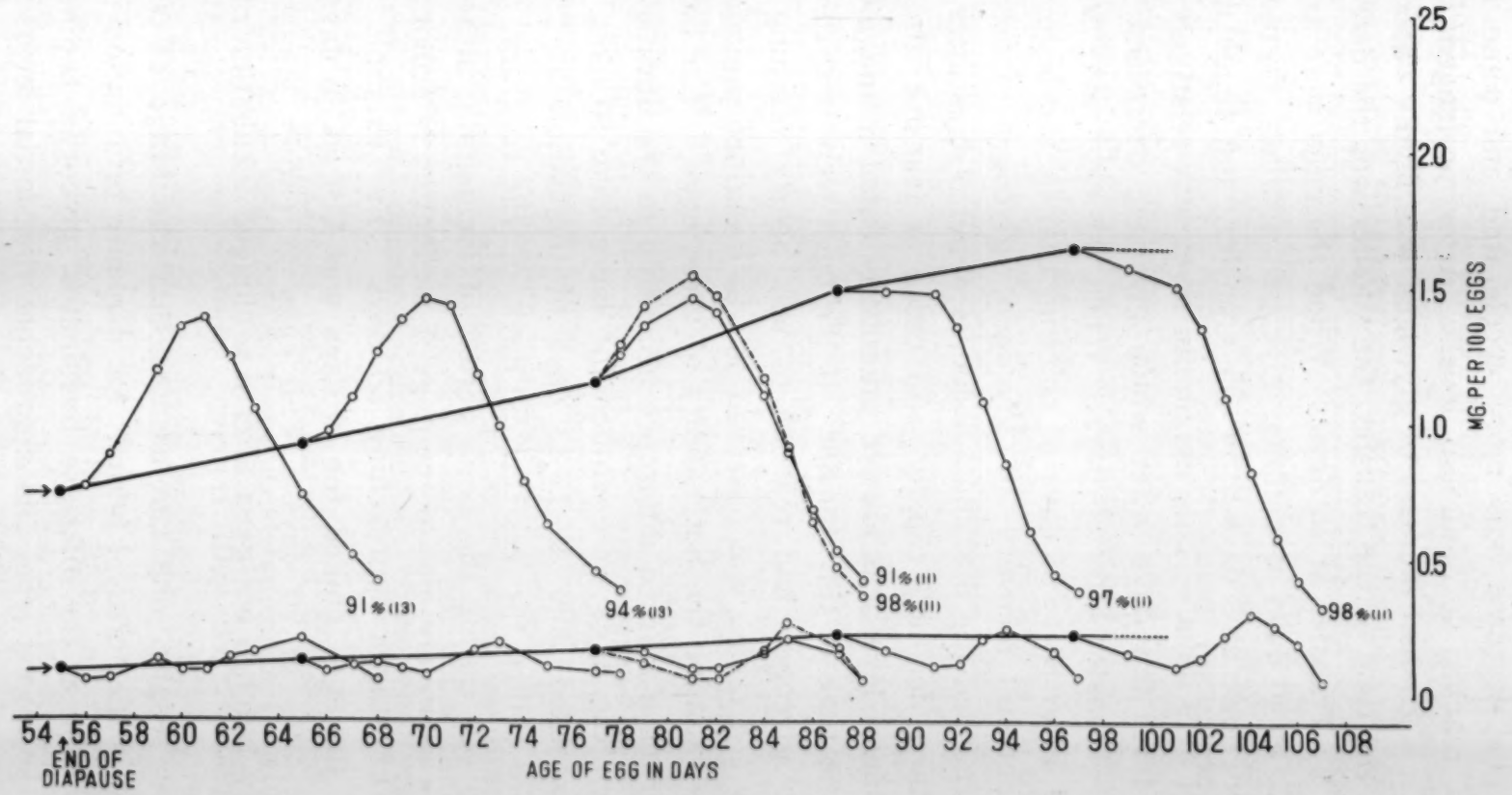


Fig. 4. Changes in content of glycogen and glucose of 'diapause' egg which was transferred to 5° C. after 2 days at 25° C. ○: eggs at 25° C., ●: eggs at 5° C., —·—: the changes when the eggs were treated with HCl. Each figure in parenthesis indicates number of days of incubation at 25° C. Figures in percentage represent hatchability.

chilling, glycogen decrease was more accelerated and some time later glycogen reached a minimum level. After prolonged incubation at 25° C., a gradual increase of glycogen was finally observed. The later was the time of the transfer, the smaller was the decrease of glycogen. 2) Transfer at 40 days. When the eggs were transferred to 25° C. at 40 days of chilling, glycogen scarcely decreased, but began to increase at 3 days after the transfer, and reached the maximum level at 9 days. This maximum level was relatively low and persisted for a few days, after which glycogen decreased progressively. 3) Transfer after 50 days. When the eggs were transferred to 25° C. after 50 to 55 days of chilling, that is, the beginning of the spontaneous recovery of glycogen, glycogen increased promptly and reached the maximum value at about 5 days after the transfer and then declined rapidly. In cases of transfer after 50 days, the maximum level was always practically equal in magnitude regardless of the time of the transfer. If the eggs were transferred to 25° C. when glycogen had reached or was nearly reaching the maximum, only a decrease was observed as was described previously.

Now let us examine the hatchability of these eggs. The hatchability also showed a marked difference according to the duration of chilling. When the eggs were chilled for more than 50 days, the hatchability exceeded 90% at 11–13 days of incubation at 25° C. When they were chilled for less than 50 days, on the contrary, they hardly hatched out during the same period of incubation, although they did so in a relatively high percentage if incubation is prolonged, especially at the stages approaching 50 days. For example, the hatchability of the eggs chilled for 40 days was 17% at 15 days of incubation and 74% at 20 days. The eggs at earlier stages of chilling scarcely hatched out even after a prolonged incubation of 50 days or more.

From these results, one can assume that the stage at 50–55 days of chilling is the 'critical period' for diapause termination, and further that as soon as the diapause terminates, glycogen begins to increase from the lowest level and approaches the 'initial level'. In order to confirm this interpretation, the following experiments have been carried out.

Firstly, transfer to 25° C. was superimposed on HCl treatment. The eggs before the critical period were treated with HCl to break

diapause and subsequently incubated at 25° C. The changes of glycogen in these eggs are shown by the chain lines in Fig. 4. The curves indicate that glycogen increased abruptly almost immediately after HCl treatment, as was the case with the eggs transferred from 5° C. to 25° C. after the critical period, except a slight initial decrease when the acid treatment was given at the early stages of chilling. On the other hand, after the critical period, for example, at 75 days of chilling, the acid treatment became less effective for the course of glycogen shift and no marked difference was found between the treated and non-treated eggs.

Secondly, the eggs were treated with HCl at 30 days of chilling and held at 5° C. even after the treatment. In this case, glycogen increased slowly and reached the maximum level at about 30 days after the acid treatment (see Fig. 5). This behavior of glycogen is practically similar to that observed among the eggs kept at 5° C. after the critical period (cf. Fig. 4).

The above results indicate clearly that the termination of diapause is invariably accompanied by the recovery of glycogen, irrespective of the means for breaking diapause. Glycogen increases more rapidly at 25° C. than at 5° C., suggesting that the termination of diapause evokes some chemical reaction which results in the restoration of glycogen. In this connection, it is to be noted that the restoration of glycogen is to be regarded as directly associated with the termination of diapause *per se* but not with the subsequent process of post-diapause development, since embryogenesis hardly proceeds at 5° C.

Before proceeding further, it should be recalled that the eggs kept at room temperature from early summer to winter, showed a relatively low hatchability, even if they were incubated at 25° C. after the initiation of glycogen recovery (see Fig. 2). This fact seems to be contradictory to the view described above, but the discrepancy may be explained by the following consideration. The termination of diapause does not take place uniformly in the eggs kept in the laboratory for long period where temperature must fluctuate. Under such condition, the eggs would show a relatively low hatchability on subsequent incubation at 25° C. even after the glycogen curve has begun to rise, because the curve does not represent the real amount of glycogen within each egg, but it shows only

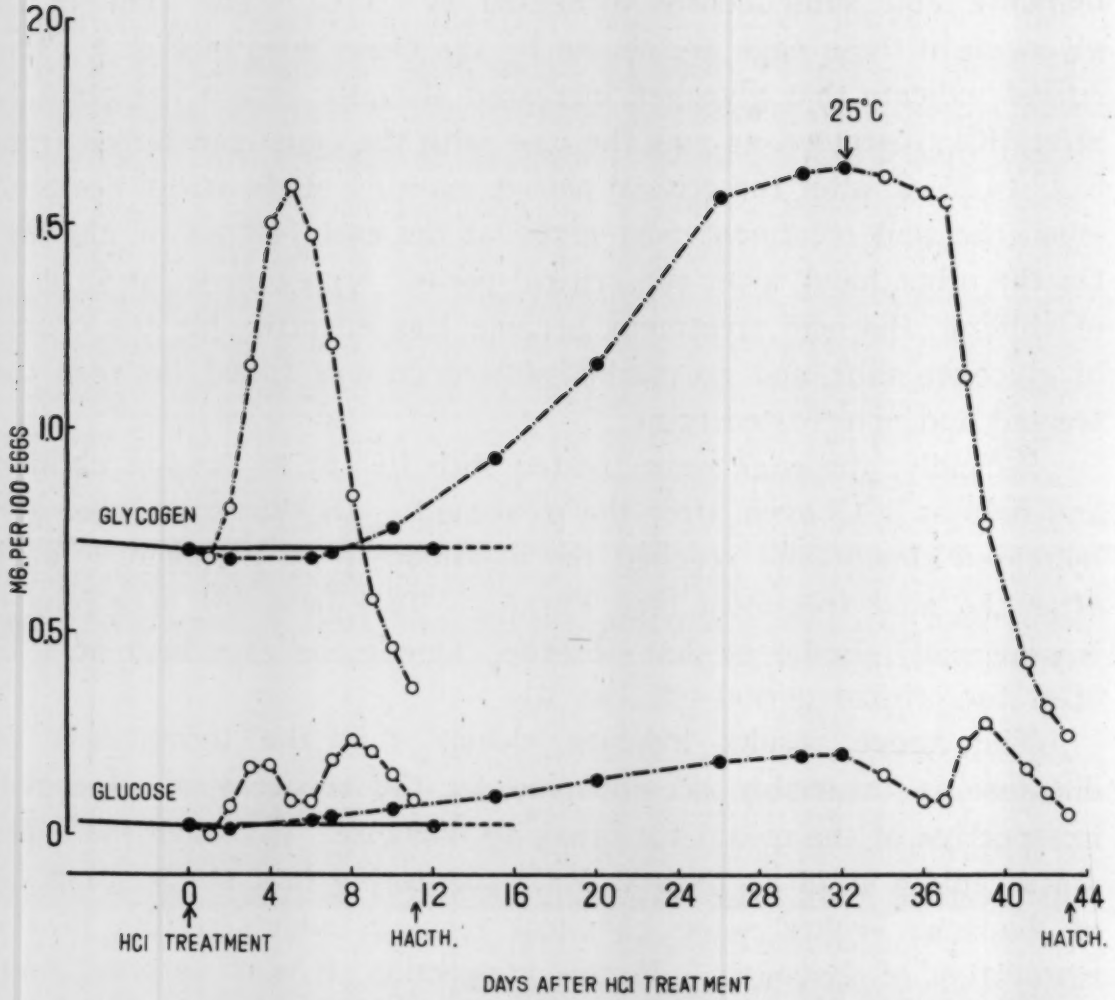


Fig. 5. Changes in glycogen and glucose content after HCl treatment of 'diapause' egg kept at 5° C. for 30 days (—·—). ——: eggs without HCl treatment, ○: eggs at 25° C, ●: eggs at 5° C.

the average value of glycogen content of the eggs analysed.

Turning to the change of glucose, it is apparent from Figs. 2 and 4 that glucose, which appeared at the onset of diapause, decreased gradually with the fall of glycogen and increased again in paralell with the recovery of glycogen up to the maximum value (0.22 mg. per 100 eggs). Thus, the sum of glycogen and glucose at the maximum, in terms of glucose, amounted to 1.85 mg. per 100 eggs. This value corresponds to 88% of the 'initial amount' of glycogen in terms of glucose. The glucose curve showed two peaks when the eggs were transferred from 5° C. to 25° C. after the critical period or after being treated with HCl. The first peak was observed after the transfer and the second one appeared at 2 to 3 days before

the hatching. The latter peak is regarded as the same as that found in 'non-diapause' egg.

Is glycogen consumed during the diapause period?

As described previously, when diapause sets in, the glycogen content of the eggs decreases markedly and as soon as diapause is brought to an end by low temperature or by HCl treatment, it begins to increase and approaches the 'initial level'.

Now the question arises as to the fate of glycogen during the diapause period. NAKATA (1952) has suggested that the main bulk of glycogen is consumed as an energy source during the early period of diapause, and it is resynthesized from fat and protein in the later period of diapause. However, from the following reasons enumerated below, it appears probable that glycogen is not consumed, but is converted to other substances and is formed again from the substances after the termination of diapause: 1) Since the initial decrease of glycogen of 'diapause' eggs is far more rapid in contrast to 'non-diapause' eggs to reach a certain developmental stage, it is unlikely that the diapausing eggs consume more glycogen as fuel than the developing eggs. 2) The time when glycogen begins to decline markedly in 'diapause' eggs coincides closely with the time when oxygen uptake of the eggs begins to fall rapidly. 3) The maximum level to which glycogen (to be exact, the sum of glycogen and glucose) attains at 5° C. after the termination of diapause is nearly the same as the 'initial level' of glycogen. 4) In the eggs kept at 25° C. for 2, 10 or 20 days after oviposition respectively and then transferred to 5° C., glycogen declined to different minimum levels. Nevertheless, the maximum levels attained after the termination of diapause are approximately equal.

In addition to the above observations, the following experiments indicate conclusively that the marked decline of glycogen with the onset of diapause is not attributable to its consumption as fuel. First, the 'diapause' eggs were kept at 25° C. immediately after the oviposition and the oxygen uptake of 1 gram of the egg was manometrically measured every day during the period from 1 to 7 days after oviposition. At the same time, the amount of oxygen which was required to oxidize glycogen, was calculated under the assumption that the observed decrease of glycogen is due to its com-

Table 1. Comparison between oxygen uptake and the decrease of total carbohydrate content of 'diapause' egg during the period from 1 to 7 days after oviposition

Duration of experiment as expressed in number of days after oviposition	(A) O_2-uptake μl./g.* of egg/day	Decrease of carbohydrate mg./g. of egg/day	(B) Theoretical amount of O_2 required for oxidation of carbohydrate (left column)	A : B
1→2	1,870	11.0	8,250	1:4.4
2→3	805	6.2	4,650	1:5.8
3→4	330	3.6	2,700	1:8.2
4→5	265	1.6	1,200	1:4.6
5→6	250	1.4	1,050	1:4.2
6→7	230	1.3	980	1:4.3
1→7	3,750	25.1	18,830	1:5.0

* Weight on the 1st day.

Table 2. Comparison between the decrease of dry-weight and that of total carbohydrate content of 'diapause' egg during the period from 1 to 10 days after oviposition

Stage	Exp. No.	Fresh weight (mg.)	Dry weight (mg.)	Carbohydrate mg./g.* of egg
1 day after oviposition	1	1,000.0	366.0	
	2	1,000.0	367.0	
	3	1,000.0	367.0	
	Average	1,000.0	366.7	39.0
10 days after oviposition	1	986.0	361.0	
	2	986.0	362.0	
	3	986.0	361.0	
	Average	986.0	361.3	10.7
Difference between 1 and 10 days	Average	14.0	5.4	28.3

* Weight on the 1st day.

plete oxidation. As shown in Table 1, the calculated values always exceeded the actual oxygen uptake considerably. When compared in total, the ratio between the two values was about 5. Second, the decrease of dry weight of the eggs during the period from 1 to 10 days after oviposition was measured. For this purpose, several batches of exactly 1 gram of the eggs were prepared. A half of them was dried immediately and another half was dried after 10 days. As indicated in Table 2, the dry weight of 1 gram eggs at 1 day

after oviposition was 366.7 mg., and that at 10 days was 361.0 mg. In other words, the decrease in dry weight during the above period was only 5.7 mg. per g. of egg, whereas the decrease in glycogen content during the same period amounted to 28.3 mg. per g. of egg $\left(\text{to be exact, } 28.3 \text{ mg.} \times \frac{\text{M.W. of } C_6H_{10}O_5}{\text{M.W. of } C_6H_{12}O_6}\right)$. Thirdly, the change in lactic acid content of the egg during the period from 1 to 30 days after oviposition was measured. As shown in Fig. 6, lactic acid was not found in detectable amount for the first 1 day after oviposition, namely, during the pre-diapause period, but it appeared concurrently with the beginning of the marked decline of glycogen. Thereafter, lactic acid increased gradually up to a maximum value at about 10 days after oviposition, after which it began to decrease slowly. Even at its maximum level, however, the amount of lactic

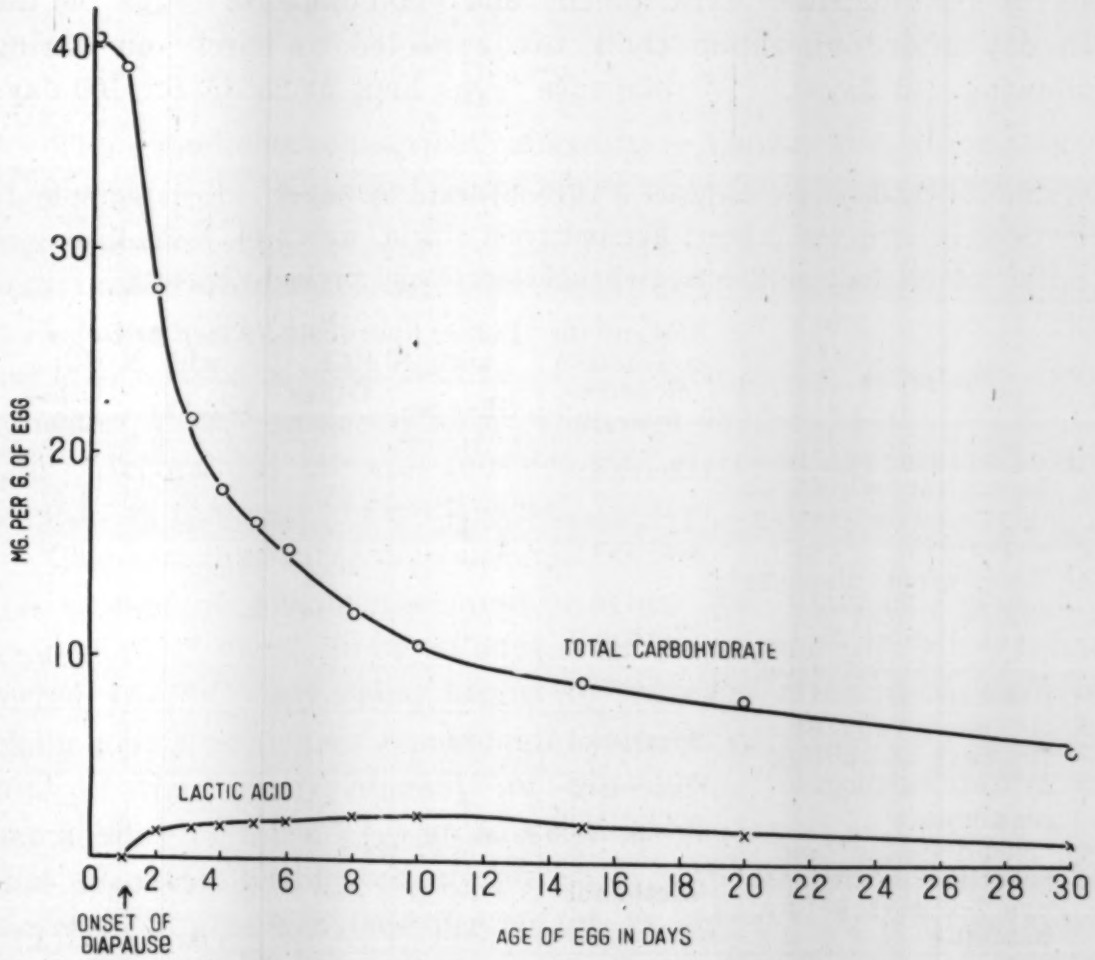


Fig. 6. Changes in content of total carbohydrate and lactic acid of 'diapause' egg during the period from 1 to 30 days after oviposition. Total carbohydrate quantity is represented in terms of glucose.

acid calculated corresponds to only 7% of the decrease in glycogen content. Besides, lactic acid never increased after 10 days, although glycogen continued to decrease until 30 days.

From the above results it is apparent that neither respiration nor glycolysis can account for the initial decrease in glycogen content with the onset of diapause.

Qualitative analysis of carbohydrates by paper chromatography

The identification of carbohydrate of the eggs have been carried out by paper chromatography on the following materials: 1) 'diapause' eggs on the 2nd day after oviposition (glycogen decreasing-stage). 2) 'diapause' eggs reactivated by HCl and incubated at 25° C. for 3 days (glycogen-increasing-phase). 3) 'diapause' eggs kept at 5° C. for 100 days (glycogen maximum). 4) 'diapause' eggs on the 8th day of post-diapause development and 'non-diapause' eggs on the 7th day after oviposition (both are expected to hatch out during following 2-3 days). 5) 'diapause' eggs kept at 25° C. for 100 days

Table 3. Qualitative analysis of carbohydrate by paper chromatography. Solvent of development: butanol, acetic acid, water (4:1:2), Spot detection: aniline hydrogenphthalate and orcinol reagents

Materials	Solvent for extraction of carbohydrate	Before hydrolisis with N HCl		After hydrolysis with N HCl	
		Glucose	Other sugar	Glucose	Other sugar
1) 'Diapause' egg at 2 days after oviposition (glycogen decreasing phase)	Methanol	+	−		
	Trichloro-acetic acid	+	−	++++	−
2) Reactivated 'diapause' egg at 3 days of incubation at 25° C. (glycogen increasing phase)	Methanol	+	−		
3) 'Diapause' egg at 100 days of chilling at 5° C. (glycogen maximum)	Methanol	+	−		
	Trichloro-acetic acid	+	−	++++	−
4) 'Non-diapause' and reactivated 'diapause' egg 2-3 days before hatching	Methanol	+	−		
5) 'Diapause' egg at 100 days after oviposition at 25° C. (glycogen minimum stage)	Methanol	−	−		
	Trichloro-acetic acid	−	−	+	−

after oviposition without chilling (glycogen minimum stage). Except in 5), glucose was found to be at high levels (see Figs. 1, 2, 3 and 4).

The results are shown in Table 3, which indicates that glucose is the only monosaccharide found in the eggs throughout diapause and embryogenesis, and also that no oligosaccharides are present in the eggs at any stages so far as they are detected by aniline hydrogenphthalate and orcinol reagents. The table reveals further that the polysaccharide extractable with hot 5% trichloroacetic acid consists of glucose only. This fact proves that the polysaccharide found in the egg must be glycogen. Such findings by paper chromatographic techniques offer a basis for considering that the carbohydrate value in the eggs obtained by the colorimetric method of MENDEL *et al.* (1954) represents the glucose amount.

DISCUSSION

The experiments described above have shown that the changes of glycogen in 'diapause' eggs differ considerably from those in 'non-diapause' eggs. In the former, glycogen begins to decrease very rapidly at about 1 day after oviposition, whereas, in the latter, it decreases gradually till about half way of development. The initiation of the marked decline of glycogen in the 'diapause' eggs coincides closely with the time when the oxygen uptake begins to fall. Accordingly one can consider that glycogen decreases rapidly as soon as the eggs enter diapause.

The termination of diapause also has an intimate relation with the change of glycogen content. When the 'diapause' eggs are kept at 25° C. for 2, 10 or 20 days after oviposition and then transferred to 5° C., glycogen begins to increase after 50–55 days of chilling and attains to a maximum amount, corresponding to about 90% of the 'initial amount' of glycogen. The examination of hatchability of these eggs on subsequent incubation at 25° C. reveals that diapause terminates concurrently with the beginning of the increase of glycogen (see percentage in Fig. 4). It is to be noted that the incubation at 25° C. of the eggs after 50 days of chilling induces a rapid increase of glycogen, whereas shorter chilling fails to induce such an increase. Furthermore, when diapause is broken

by treating the diapausing eggs with HCl, glycogen shows a similar rapid increase as observed in the eggs chilled for more than 50 days. From these observations one can conclude that as soon as diapause terminates, glycogen begins to increase and restores the 'initial level' of glycogen nearly completely.

Now the question arises as to what happens to the glycogen during diapause period, since the disappearance of glycogen cannot be explained on the basis of its change into glucose, as both increase or decrease simultaneously. As was described previously, since it is apparent that glycogen is not consumed as an energy source, the main bulk of it must be converted into other substance or substances. It should be mentioned in this connection that no phosphoric esters accumulate during diapause period, but they rather decrease (CHINO, 1956). Furthermore, the chromatographic result, presented above, indicates that the eggs in diapause state do not contain any carbohydrate other than glucose and glycogen.

The possibility that glycogen might be converted into some other form highly resistant to acid hydrolysis, is also denied by the following experiment; the eggs in diapause state were ground with 5% trichloroacetic acid and then centrifuged. The supernatant was hydrolysed by 35% HCl for 8 to 12 hours with no increase of sugar at all. There remains another possibility that in diapause state glycogen is bound firmly to other substance such as protein, so that it is hardly extracted with trichloroacetic acid. However, the presence of glycogen in the residue after the extraction with trichloroacetic acid could not be demonstrated. even on digestion with alkali.

From these results, one can conclude that substance into which the main bulk of glycogen converts itself is neither sugars nor their polymers nor their phosphoric esters nor lactic acid.

Pursuing further the metabolic fate of glycogen during diapause, the author has recently obtained result indicating that glycogen is converted into two kinds of polyhydric alcohols, namely, sorbitol and glycerol. A more detailed account of this finding and related experiments will be published in the next paper.

SUMMARY

The changes of glycogen and glucose in developing and dia-

pausing eggs of the silkworm, *Bombyx mori*, have been investigated. The following results were obtained.

1) In 'non-diapause' eggs, glycogen decreased gradually at first, then rapidly during embryonic development.

2) In 'diapause' eggs, glycogen decreased slowly in the pre-diapause period, and it began to decrease abruptly as soon as the eggs entered diapause. It reached a minimum level (nearly one tenth of the 'initial level' in pre-diapause) at about 30 days after oviposition if kept at 25° C. The minimum level was maintained throughout the rest of the diapause period.

3) If the 'diapause' eggs were converted into non-diapause type by treating with dilute HCl at 20 hours after oviposition before the onset of diapause, the marked decline of glycogen characteristic to diapause was not observed; instead, the course of change in glycogen became quite similar to that in natural 'non-diapause' eggs.

4) When the eggs were incubated at 25° C. after diapause was broken either by chilling or by hydrochloric acid treatment, glycogen began to increase rapidly and at half way of development it recovered its 'initial level' nearly completely. Thereafter it decreased rapidly until the hatching time. The restoration of glycogen content is to be regarded as directly associated with the 'break' of diapause *per se* and not with the subsequent process of embryogenesis, because the recovery of glycogen could proceed even at 5° C. at which temperature embryogenesis was suppressed almost completely.

5) The amount of glucose found in the eggs was very low as compared with that of glycogen. In 'non-diapause' eggs, glucose first appeared at 1 day after oviposition and increased with embryogenesis, reaching a maximum value at 2 days before the hatching time. In 'diapause' eggs, glucose was found in a small amount at the onset of diapause, but it soon disappeared. It reappeared after the termination of diapause and increased with the rise of glycogen content. The change of glucose during post-diapause development was essentially similar to that of 'non-diapause' eggs.

6) The marked decline of glycogen with the onset of diapause could not be accounted for either by respiration or glycolysis. Evidences have been presented that during the diapause period glycogen is converted into some substance or substances other than lactic acid, sugars and their polymers.

Acknowledgement

The author wishes to thank Professors Y. Akita and T. Fujii for their kind advices and valuable criticisms. Thanks are also due to Professor K. Dan for helpful criticism and reading the manuscript, and finally to Dr. S. Fukuda for his kindness of supplying the materials.

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ON THE MECHANISM OF THE DORSALIZATION IN THE ECTODERM OF *TRITURUS* GASTRULAE CAUSED BY PRECYTOLYTIC TREATMENTS*

I. CYTOLOGICAL AND MORPHOGENETIC EFFECTS OF VARIOUS INJURIOUS AGENTS

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INTRODUCTION

As demonstrated by HOLTFRETER (1944, 1945, 1947) the ectoderm isolated from the early gastrula of *Triturus torosus* is capable of differentiating into neural tissue without any inductive influence from the organizer, when it is subjected to some disaggregating media such as Ca-free, alkaline, acid solutions or 10% alcohol. He concluded that the neuralization after these treatments is initiated by an increase in permeability of the cell membrane, which is followed by a mild cytolysis. Under such conditions the "neuralizing agent" may be released from a certain cytoplasmic component (HOLTFRETER, 1947, 1948).

Later YAMADA (1950*a*) showed that an appropriate shock treatment with ammonia leads the gatrula ectoderm of *Triturus pyrrhogaster* to differentiate archencephalic structures including brains, eyes, noses, and mesenchyme, and that the same treatment of the medioventral marginal zone evokes the differentiation of notochords and

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somites. He designated these effects of ammmonia treatment as "dorsalization".

In general the term dorsalization is applied, if in a developing system a change is introduced, which affects the subsequent differentiation of the system in such a way that the dorsal structures are produced at the expense of the ventral ones (YAMADA, 1950 *a*, *b*). If applied in particular to the presumptive ectoderm of *Triturus* gastrula with an inherent tendency to differentiate the epidermis which is normally derived from the ventral part of the ectoderm, dorsalization denotes the change which leads to the differentiation of neural tissue, sense organs, mesenchyme or melanophores, which all are normally derived from the dorsal part of the ectoderm. Some attempts are made to elucidate the nature of dorsalization in the present series of papers.

The immediate effects of the above cited injurious treatments involve some visible alteration of the embryonic cell, *i.e.*, amoeboid movement of the disaggregated cells, endoplasmic sol-gel transformation, and so on (HOLTFRETER, 1945, 1946 *a*, *b*, 1947, 1948 *a*). It is possible that some of these processes are causally connected with dorsalization. In the pressent paper the immediate effects of various injurious agents on the cytological structure of embryonic cells were studied in relation to their effects on the differentiation which the treated cells undergo on further culture.

MATERIAL AND METHODS

A piece of the presumptive ectoderm was isolated from the early gastrula of *Triturus pyrrhogaster* (Stage 11 after OKADA and ICHIKAWA). The size of the isolate varied from 0.8 to 1.5 mm. in diameter. Immediately after isolation, the ectodermal pieces were immersed in the following media.

Ammonia solution: Prepared immediately before use by adding 0.01 cc. of ammonia (20%) to 10 cc. of unbuffered HOLTFRETER solution. Its pH was estimated to be *ca.* 12.0.

Sodium hydroxide solution: 0.1 cc. of 1 N sodium hydroxide added to 100 cc. of unbuffered HOLTFRETER solution (pH *ca.* 12.0).

Hydrochloric acid solution: Prepared by giving 0.1 cc. of 1 N hydrochloric acid to 100 cc. of HOLTFRETER solution (pH *ca.* 2.0).

Urea solution: M/8 urea mixed with an equal volume of HOLTFRETER solution (M/16 urea, pH 7.0).

Sodium thiocyanate solution: Prepared by mixing equal volume of M/16

sodium thiocyanate and HOLTFRETER solution (M/32 sodium thiocyanate, pH 7.5).

"Ca-free" solution: HOLTFRETER solution from which calcium chloride was omitted. It was buffered with sodium bicarbonate usually at pH 6.9–7.1, exceptionally at pH 8.0 or 8.8. Probably a small quantity of calcium was present in the medium. When its thorough removal was desired, 10 cc. of M/16 sodium oxalate was added to 90 cc. of "Ca-free" HOLTFRETER solution.

Sucrose solution: M/8 sucrose in distilled water (pH 6.0).

Lithium chloride solution: M/16 lithium chloride in distilled water (pH 7.0).

Alcohol solution: 15% solution of ethyl alcohol in HOLTFRETER solution (pH 7.0).

When an ectodermal piece was placed in any of these solutions, it was disintegrated into a heap of separate cells, the velocity of disintegration being different for different media.

For observation *in vitro*, the disaggregated cells were transferred with a fine pipette onto a slide, and observed under a phase contrast microscope.

Disaggregated or reaggregated cells were cultured in a small Stender dish containing *ca.* 25 cc. HOLTFRETER solution (pH 6.5–7.2). Operation and cultivation were done under sterile conditions. Effort was made to keep sterile conditions also during *in vitro* observation under a microscope.

For histological observations the explants were fixed with BOUIN's fluid after 7 to 10 days of culture at 18° C., sectioned at 12 μ, and stained with MAYER's hemalum and "picro-Blauschwarz".

RESULTS

I. Effects of disaggregation with "Ca-free" HOLTFRETER solution

Disaggregation of the piece began *ca.* 10 minutes after immersion in the "Ca-free" medium. Although the velocity of disaggregation varied considerably, the majority of the pieces were completely disaggregated into single spherical cells within 40 minutes to 1 hour. With the progress of disaggregation, a very small amount of mucoid substance was released around the cell mass. When perfectly disaggregated, the cells were practically non-adhesive. However on adding a small amount of M/24 calcium chloride solution to the culture medium to give the concentration of calcium chloride comparable to that of HOLTFRETER solution (M/1,000), they adhered each other to form reaggregates whose number and size varied with the extent of dispersion of cells. The solid reaggregate formed was further transferred to the second dish with HOLTFRETER solution and cultured. After *ca.* 1 hour's or less exposure to the "Ca-free" medium and subsequent treatment with the Ca-containing medium,

a nearly perfect reaggregation was attained. However a further exposure to the "Ca-free" medium diminished the ability of cells to reaggregate in the Ca-containing medium. After 12 hours' exposure, this ability was completely lost, and even an addition of excess calcium ions to the medium could not induce reaggregation, although the cells were alive for a few days.

Microscopic observation in vitro

With a phase contrast microscope the following parts could be distinguished in the cells, separated by the "Ca-free" medium (Fig. 1): (1) The central sac of the semifluid endoplasm containing the nucleus, yolk platelets, liposome, lipochondria, and pigment granules. (2) A thin layer which encloses the above noted endoplasmic mass and is distinguished by its brightness under the dark-contrast phase microscope ("plasmagel" of HOLTFRETER). (3) An outer shell of the ectoplasm which is free from visible particles, when no active protoplasmic movement is occurring. (4) A thin highly refractive surface membrane (protoplasmic membrane). These observations perfectly confirm the earlier report of HOLTFRETER (1946*b*, 1948*b*).

After disaggregation of the explant, the ectoplasmic shell of free cells appeared enlarged. After *ca.* 1 hour, the cell surface began to

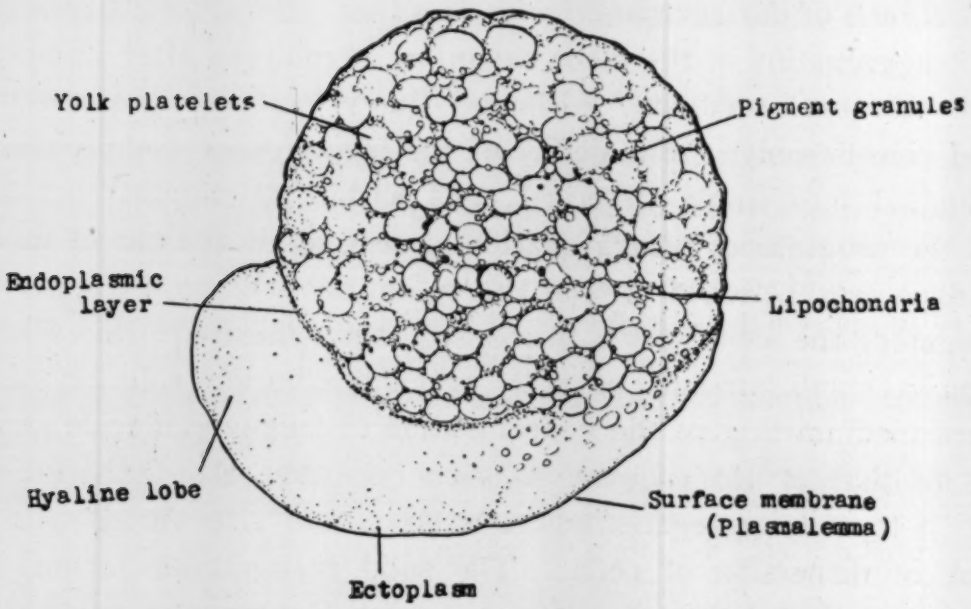


Fig. 1. The structure of an ectodermal cell isolated from the gastrula (2 hours after exposure to "Ca-free" HOLTFRETER solution).

form a mobile hyaline cap or lobopodium, which was slowly rotating around the endoplasm. At this stage of treatment no clear change could be observed either on the outer layer of the endoplasm or in the endoplasmic mass. If the treatment is extended for 2-6 hours, strong movements of the ectoplasm and partial disappearance of the outer layer of the endoplasm could be evidenced. A transfer to the standard solution gradually suppressed the rotatory movement of the ectoplasm. The cells adhered each other and amoeboid movements ceased (Table 1 and Fig. 2).

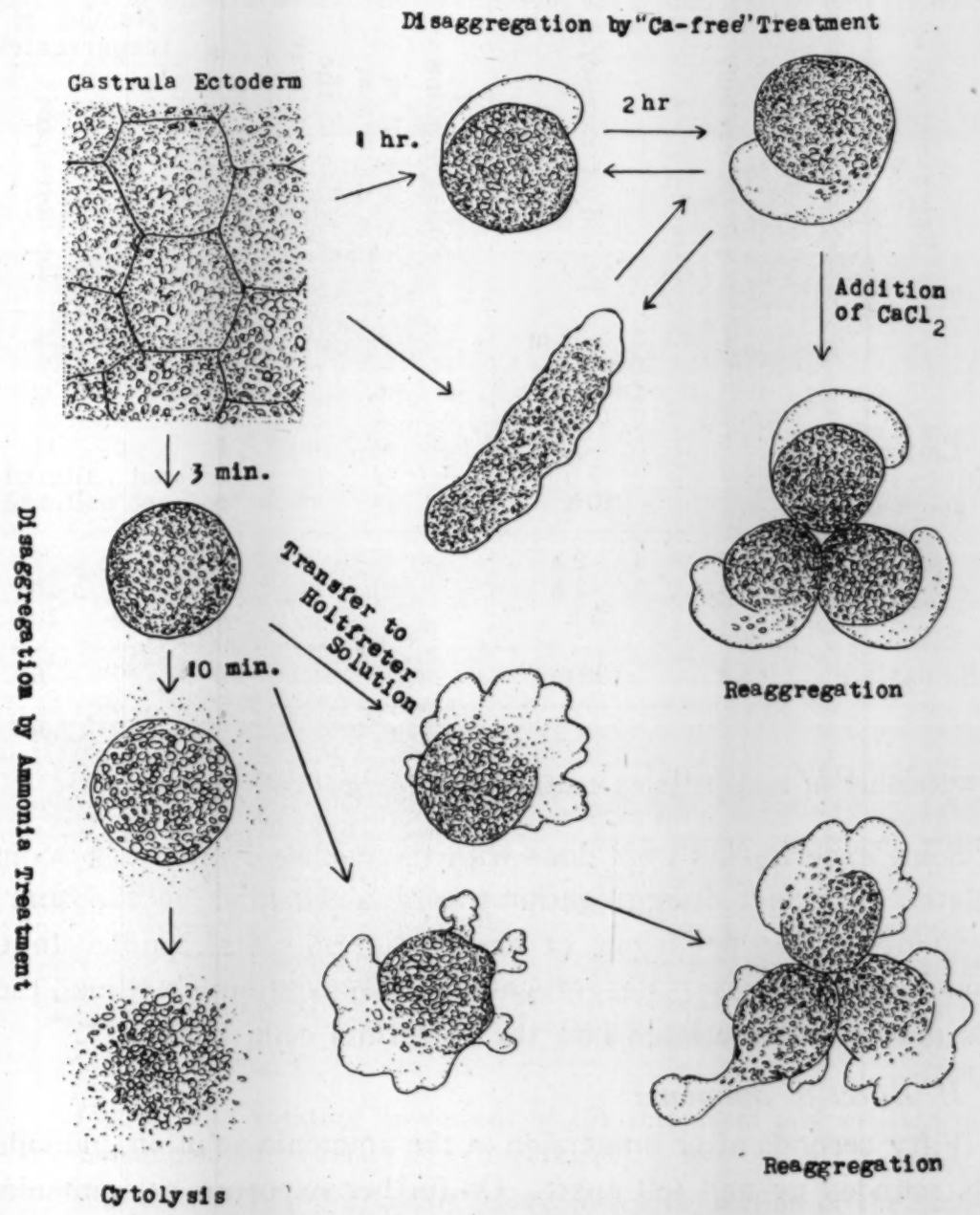


Fig. 2. Morphological changes of embryonic cells after "Ca-free" and ammonia treatment.

Morphogenetic effects

Regardless of the difference in duration of treatment, in pH (6.8–8.8), or in temperature of the medium, most of the reaggregated explants developed into the epidermis with irregular small folds. In 162 explants examined, neither neural tissue nor mesenchyme was observed (Table 1). Cytolyzed cells were sometimes found in the cavity formed within epidermal cells.

Table 1. Effects of "Ca-free" HOLTFRETER solution on cell behaviour and differentiation of the isolated ectoderm of *Triturus pyrrhogaster*

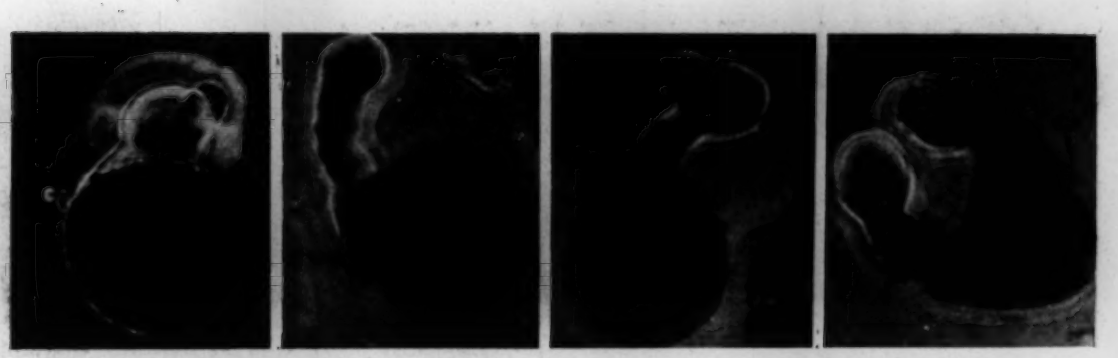
Experimental medium	pH of medium	Period of exposure	Degree of disaggregation	Movement of the ectoplasm	Solation of the endoplasmic layer	Number of original isolates	Number of reaggregates: Dorsalized	Number of reaggregates: Non-dorsalized*
"Ca-free" HOLTFRETER solution	6.8–7.4	5 –30 m	−	−	−	27	0	29
		30 m–1 h	+	−	−	29	0	26
		1 –2 h	++	+	±	33	0	32
		2 –3 h	++	+	+	15	0	25
		3 –3.5 h	++	++	++	5	0	11
		5 h	++	+++	+++	5	not cultured	
		10 h	++	+++	+++	5	not cultured	
	8.0	1 –2 h	++	+	+	15	0	9
	8.8	1 –2 h	++	++	+	14	0	20
		3 h	++	++	++	8	0	10
Na-oxalate	8.8	30 m	++	++	++	6	0	6
							0	168

* Number of reaggregates containing epidermal cells alone.

Some experiments were done with the medium containing sodium oxalate. A perfect disaggregation was obtained after an exposure of 30 minutes. The behaviour of embryonic cells was similar to the above-mentioned case. Reaggregated in Ca-containing medium, these cells always differentiated into the epidermal cells (Table 1).

II. Effect of ammonia

Fifty seconds after immersion in the ammonia solution, individual cells rounded up and fell apart. On further exposure to ammonia, a large amount of slimy substance was released from the disaggregat-



a *b* *c* *d*

Fig. 3 *a–d.* Successive changes of pseudopodia on the surface of an isolated cell after a shock treatment with ammonia. Dark-contrast. ×320.

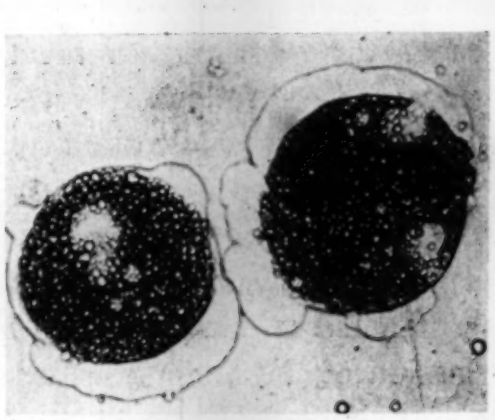


Fig. 4

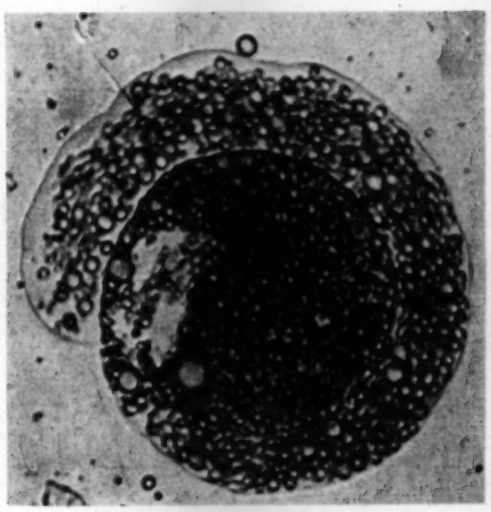


Fig. 5



Fig. 6

Fig. 4-6. Morphological changes of embryonic cells by a shock treatment with ammonia solution.

Fig. 4. The movement of the ectoplasm in the cells flattened on the glass surface (3 hours after treatment). ×320.

Fig. 5. The rotatory movement of the ectoplasm and solation of the pseudopodium (3 hours after treatment). ×400.

Fig. 6. Active protoplasmic movements with solation of the endoplasm. ×300.

ing cell-mass and a complete disintegration of the cell-mass ensued within 5 minutes.

Microscopic observation in vitro

At the moment of immersion in the solution of ammonia, the ectoplasmic layer was extremely thin and tightly attached to the endoplasm. An exposure of more than 5 minutes caused, in most cases, solation of the endoplasmic layer as well as swelling of the whole cell. The endoplasm became more transparent and granular, indicating small particles in active BROWNIAN movement. However the amoeboid movement hardly occurred in ammonia. When transferred to the standard medium after 2 minutes' exposure* to ammonia, the ectoplasmic layer soon began the rotating movement and protruded the hyaline caps. The changes were followed by the sol-gel transformation of the endoplasmic layer. Meanwhile other hyaline protrusions grew here and there on the cell surface, like fingers stretching out in all directions, sometimes fusing to form a large tongue-like protrusion. All these protrusions showed active expansion and contraction, and were frequently pinched off from the cell body. Subsequently strong sol-gel transformation of the endoplasm was observed. The protoplasmic movements exhibited in ammonia-treated cells were much more intensive than those of the cells treated with the "Ca-free" solution (Fig. 2–6).

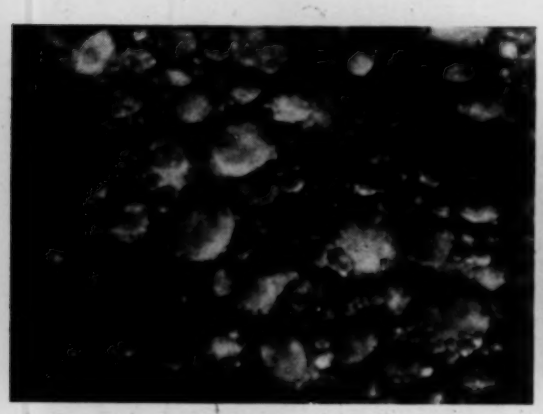


Fig. 7



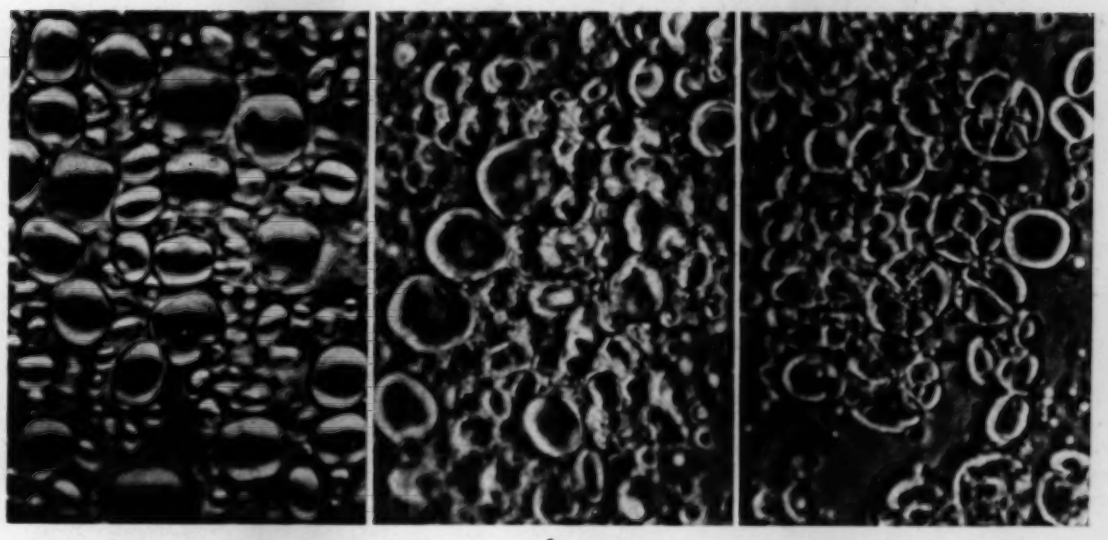
Fig. 8

Fig. 7-8. A surface view of disaggregated embryonic cells. Yolk platelets and lipochondria are visible. Dark-contast. ×800.

Fig. 7. In "Ca-free" HOLTFRETER solution.

Fig. 8. In the ammonia solution (30 minutes after immersion).

* This period was optimum for dorsalization.



a *b* *c*

Fig. 9 *a-c.* The effect of ammonia on the granules of the endoplasm. The granules kept in the ammonia solution are shown after various time intervals. Dark-contrast. ×800.

a. Immediately after the exposure.
b. Fourty minutes later. Lipochondria are disintegrating.
c. Two hours after the exposure. A cleavage of yolk platelets.

After 20 minutes or more of exposure to ammonia, the lipochondria were the first to break down. Then the yolk platelets swelled up and their spherical contour began to assume an irregular shape, suggesting a surface breakdown. Such changes were not demonstrated in cells exposed to the "Ca-free" solution (Fig. 7–9).

As noted above, a large amount of slimy substance was released from ectoderm cells by prolonged immersion in the ammonia solution. The substance which was transparent, refractive, viscous, and remarkably sticky in alkaline solutions, assumed in neutral solutions a whitish, fibrous appearance and became less sticky.

Morphogenetic effects

Before the complete disaggregation (2-3 minutes), the loose cells were transferred to HOLTFRETER solution (pH 6.9–7.2) for reaggregation and were cultured as usual. In most cases those explants became compact spheroids, or membranous disks, discharging a considerable number of free cells.

On histological examination, a considerable number (71%) of the explants showed differentiation of neural tissue or of mesenchyme

(Table 2 and 3, Fig. 10). Although in some explants brain- and nose-like structures could be identified, the majority of neural structures were unorganized. Two explants (3%) contained mesenchyme without neural tissue, while remaining 22 explants revealed only epidermis (29%). The results obtained here is principally in accord with those reported by HOLTFRETER (1945) and YAMADA (1950 *a*). On the other hand, when the isolated ectoderm was subjected to complete disaggregation by a prolonged ammonia treatment (6–15 minutes), and reaggregated in HOLTFRETER solution, it failed to differentiate into neural tissues, and formed a mass of epidermal cells (Table 2).

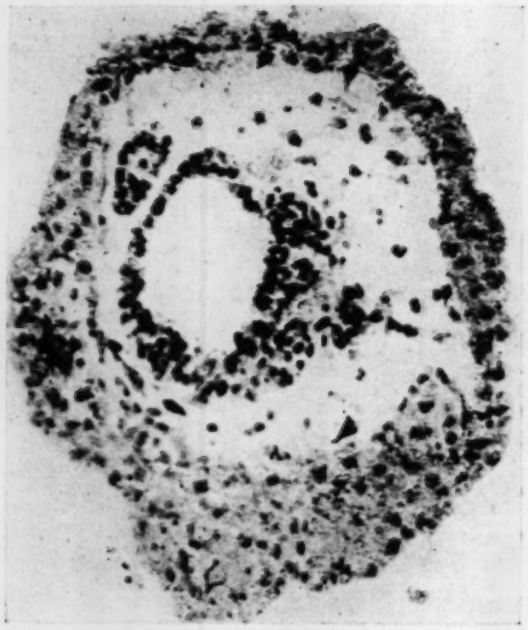


Fig. 10. Differentiation of neural vesicle, ganglion-like cell mass, melanophores and mesenchyme from the isolated ectoderm after 3 minutes' exposure to ammonia. ×100.

III. Effects of other dorsalizing agents

Results of section I and II show that the treatment with ammonia which brings about definte changes in the structure and behaviour of ectodermal cells is followed by differentiation of neural tissue and mesenchyme, while the "Ca-free" medium with only a weak immediate effect on the cells does not affect the subsequent differentiation of the explants. The question may be raised, whether a similar relationship holds for other agents with and without dorsalizing effects. In the following series of experiments, some dorsalizing agents were studied for their immediate effects on the cells.

NaOH treatment

The isolated ectoderm was completely disaggregated by treating with sodium hydroxide solution of pH 12.0 for 4 to 10 minutes. After 5 minutes' exposure, disaggregated cells were transferred to HOLTFRETER solution, where they exhibited active protoplasmic movements

Table 2. Effects of various agents on cell behaviour and differentiation of the isolated ectoderm of *Triturus pyrrhogaster*

Experimnetal solutions	pH of solutions	Time required for complete disaggregation	Period of exposure	Degree of disaggregation	Release of mucous substance	Movement of ectoplasm	Solation of endoplasmic layer	Number of reaggregates: Total number	Dorsalized*	Non-dorsalized
Ammonia	12.0	2- 5 m	1- 3 m	+	++	+++	+++	75	53	22
			6-15 m	++	+++	+++	+++	20	0	20
NaOH	12.0	4-10 m	3- 6 m	+	++	++	++	20	5	15
HCl	2.0	5-15 m	4- 7 m	++	+	++	++	19	11	8
Urea (M/16)	7.0	2- 6 m	2- 5 m	+	+++	++	+++	20	8	12
NaSCN (M/32)	8.0	10-20 m	20 m	++	+	++	++	26	5	21
"Ca-free"	6.8-8.0	30 m-1 h	5 m-3 h	++	±	+	+	121	0	121
Sucrose (M/8)	6.5	2-24 h	3 h	+	−	±	−	15	0	15
LiCl (M/16)	7.0	2-24 h	3 h	+	−	±	±	20	0	20
Alcohol (15%)	7.0	1 h	2 h	+	−	++	−	18	0	18

* See Table 3.

Table 3. Histological observations of explants treated with dorsalizing agents

Experimental solutions	Number of reaggregates studied	Total of dorsalized reaggregates	Number of reaggregates with: Brain	Nose	Neural mass	Neuroid*	Melanophore	Mesenchyme
Ammonia	75	53	11	3	27	13	8	2
NaOH	20	5	1	0	3	1	1	0
HCl	19	11	2	0	5	4	2	1
Urea	20	8	2	0	3	3	1	0
NaSCN	26	5	0	0	1	4	0	0

* Cell condensation without a clear neural character.

accompanied with expansion-contraction of the ectoplasm and sol-gel transformation of the endoplasm. A prolonged exposure of the cells to sodium hydroxide resulted in the release of a considerable amount of mucous substance and in the breakdown of yolk platelets and lipochondria.

In the other experiments, the explants treated with the same solution of sodium hydroxide were transferred to HOLTFRETER solution

before their complete disaggregation, and cultured in order to study their development (Table 2). The sections through the cultured explants revealed a weaker dorsalizing effect.

HCl treatment

After an exposure to hydrochloric acid for a few minutes, the ectoplasmic layer enlarged, and the plasma membrane appeared separated from the endoplasm. However no BROWNIAN movement of the endoplasmic granules was observed. Upon transfer to HOLTFRETER solution after 4 to 7 minutes of exposure, the rotating movement of the ectoplasm became as active as after treatments with alkalies. Also the outflow of the endoplasmic granules in the ectoplasm was very vivid. When the exposure to hydrochloric acid was prolonged, a coarse network appeared within the ectoplasmic area and the endoplasmic inclusions stopped streaming, probably indicating an irreversible coagulation in both ectoplasm and endoplasm. In this series no release of mucous substance was recognized.

A number of cell-group reaggregated in HOLTFRETER solution after treatment with hydrochloric acid for 4 to 7 minutes was cultured as usual. Dorsalization of the ectoderm occurred at a considerable frequency (Table 2 and 3).

Urea treatment

Immediately after exposure to M/16 urea, the ectodermal piece discharged much mucous substance. A few minutes later, cytolysis took place in the cells lying in the periphery of the piece. Active

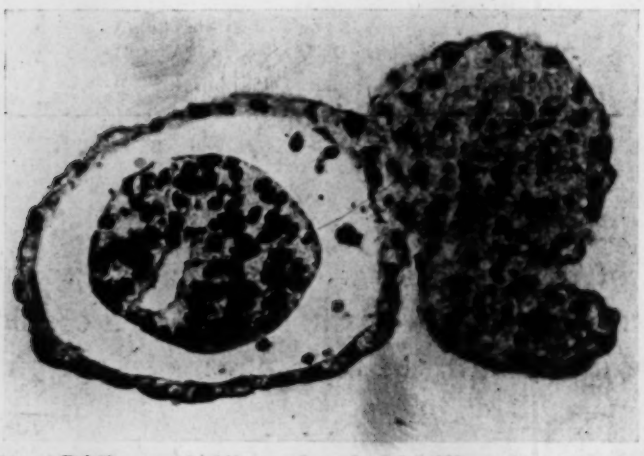


Fig. 11. Differentiation of a brain-like structure from the isolated ectoderm after an exposure to urea. ×150.

protoplasmic movements such as occurred following the ammonia treatment was observed in some of the separated cells after the transfer to HOLTFRETER solution, although observation was often disturbed by the turbidity of the medium caused by cell-debris.

Explants were exposed to urea for 3 to 5 minutes, and transferred to HOLTFRETER solution and cultured. Owing to a severe damage, only small cell-aggregates survived, which further discharged free cells during the culture. The histological sections evidenced a dorsalizing effect (Fig. 11, Table 2 and 3).

NaSCN treatment

The treatment caused a thorough disaggregation of the ectoderm within 20 minutes. The free cells produced by the disaggregation exhibited, even in the solution of NaSCN, the active protoplasmic movements, which were observable in other series only after the transfer to HOLTFRETER solution. However also in this case a transfer to HOLTFRETER solution enhanced the movement. A prolonged exposure to sodium thiocyanate, caused the increase in volume of cells without a rupture of the plasma membrane.

In the reaggregates cultured after an exposure to sodium thiocyanate a weak dorsalizing effect was observed (Table 2 and 3).

Summarizing the results obtained in this section, a brief exposure of the explants to sodium hydroxide, hydrochloric acid, urea, or sodium thiocyanate has been found to bring about dorsalization. On the other hand an appropriate treatment with these agents enhances active protoplasmic movements in the ectodermal cells accompanied with formation of various types of pseudopodia and solation of endoplasmic layer. The effects of these agents on the protoplasmic state and differentiation are comparable to those of ammonia, although in general the latter is more effective than the formers.

IV. Effects of non-dorsalizing agents

In the present section some agents without dorsalizing effects were studied for their effect on the structure and behaviour pattern of the cells (Table 2).

Sucrose treatment

Sucrose added to HOLTFRETER solution in different concentrations did not disintegrate the ectodermal explant up to two days. Only

the pure isotonic sucrose solution could partially disintegrate the ectodermal explants, although even then some of the explants showed no sign of disaggregation after an exposure of 24 hours. In this medium, disaggregated cells did not show pseudopodia, and the ectoplasmic surface attached tightly to the endoplasm. The volume of the single cell seemed to decrease during the application of sucrose, suggesting dehydration. The BROWNIAN movement in the endoplasm was apparently suppressed. After the transfer to HOLTFRETER solution, formation of pseudopodia was insignificant and sol-gel transformation at the endoplasmic layer was hardly observed. The transfer from sucrose to HOLTFRETER solution was made in steps in order to avoid the cell rupture which occurred upon a sudded transfer.

After 3 hours' exposure, the loose cells were transferred to HOLTFRETER solution and cultured. They recovered perfectly and differentiated into masses of epidermal cells arranged highly irregularly. No dorsalization was observed.

LiCl treatment

When explants were treated with a M/16 solution, no indication of disaggregation was observed for the first 2 hours. Later they began to round up into spheres, gradually fell apart, and were completely disintegrated by 3 hours. Some of the explants underwent curling, and showed no sign of disaggregation even after 24 hours. The behaviour of the free cell in this medium was similar to that in "Ca-free" HOLTFRETER solution. However in the lithium chloride solution the single cell was surviving for more than 24 hours exhibiting amoeboid movements, whereas in the "Ca-free" medium a large number of cells solated and cytolyzed within 10 hours.

Even after an exposure of 3 hours to lithium chloride, free cells perfectly reaggregated in HOLTFRETER solution and, upon further culture, differentiated into atypical epidermis. No differentiation of neural cells or mesenchyme was observed.

Alcohol treatment

Ectodermal pieces which were exposed to HOLTFRETER solution containing ethanol (15%), underwent an inverse curling, forming cups with the original internal surface facing outside. About 30 minutes later the explant began to disaggregate, and a complete disaggre-

gation was attained within 1 to 2 hours. A number of free cells formed unusually large and entirely hyaline pseudopodia, although the sol-gel transformation of the endoplasm was not detectable. Sometimes a fibrous hyaline process appeared on the surface, a condition not observed after any other treatments.

In the culture-experiment none of the reaggregates tended to neural differentiation. This was the case even if the reaggregates were obtained after an exposure to ethanol for 2 hours which led to a perfect disintegration.

Discussion

As mentioned in the introduction, the ectoderm of *Triturus torosus* differentiates neural tissues when exposed to "Ca-free" solution or 10% alcohol (Holtfreter, 1947). However in the present experiments the isolated gastrula ectoderm of *Triturus pyrrhogaster* disaggregated with the "Ca-free" medium or with 15% alcohol does not differentiate into neural tissues but forms only the atypical epidermis like the untreated explant, while the ectoderm treated with the alkaline or acid medium is shown to differentiate into neural tissues. The different behaviour of the ectoderm toward the "Ca-free" medium or alcohol in the present and Holtfreter's experiments may be ascribed to the difference in susceptibility of ectodermal cells between the two species of *Triturus*. It is likely that the ectodermal cells of *T. pyrrhogaster* are less susceptible than those of *T. torosus*, and that the exposure to the calcium deficient medium or to alcohol is injurious enough to dorsalize the ectoderm of *torosus*, but not enough to dorsalize the ectoderm of *pyrrhogaster*, while treatments with exterme pH are drastic enough for dorsalization of the ectoderm of both species. In any case the present results definitely establish that *disaggregation of the gastrula ectoderm is not the sufficient condition for dorsalization.*

Beside alkali and acid, urea and sodium thiocyanate cause neural differentiation in the presumptive ectoderm. The effects of these substances on the embryonic determination have been known under various experimental conditions. For example, a hypertrophy of the notochord in the frog embryo was induced by urea (Fautrez, 1949) or by sodium thiocyanate (Ranzi and Tamini, 1939). Furthermore

explants of the ventral marginal zone differentiated notochord when exposed to urea (LEONE, 1949) or to sodium thiocyanate (ÔGI, 1957). Thus the present results lend support to the idea suggested by YAMADA (1950*a*) that the agents which dorsalize the ventral mesoderm, can dorsalize the ectoderm as well. Similarly the dorsalizing effect of lactic acid has been demonstrated on the ventral mesoderm (KAWAKAMI and OKANO, 1955) as well as on the ectoderm (HOLTFRETER, 1947).

It is confirmed that an exposure of *Triturus* ectoderm to extreme pH, urea, or sodium thiocyanate for not more than a few minutes is sufficient to activate the whole chain of events which lead to neural differentiation. Immediately after these treatments the following changes are observed in the ectoderm cells: swelling by hydration, increase of the intracellular BROWNIAN movement, active protoplasmic movements accompanied by the various types of pseudopodia, solation of the endoplasmic layer, structural changes or breakdown of cytoplasmic inclusions. On the other hand, a short exposure to "Ca-free", sucrose, and lithium chloride solutions hardly induces such changes in ectoderm cells, and does not cause dorsalization, although a perfect disaggregation is attained. Especially the changes caused by sucrose appear to be opposite in nature to the changes induced by ammonia treatment. The results obtained thus suggest that the activation of the intensive protoplasmic movements may be in some way connected with dorsalization.

There is probably no direct causal relation between the release of mucous substance and dorsalization, since hydrochloric acid is more effective for dorsalization than sodium hydroxide, although more mucous substance appears after treatment with the latter than after that of the former.

The alcohol treatment, which does not cause dorsalization, increases the hyaline area of the ectoplasm, and enhances its undulating movement; whereas endoplasmic solation is not observable after this treatment. On the other hand endoplasmic solation is one of the common effects of dorsalizing treatments (Table 2). Thus it is not improbable that the marked solation of the endoplasm observed upon short application of dorsalizing agents is linked to the essential processes of dorsalization. It should be recalled that a similar view was earlier expressed by RANZI *et al.* (1952, 1954) on

the ground of their studies on the effect of animalizing agents* including ammonia, urea and sodium thiocyanate on the physical nature of some proteins.

The main parts of the observations made by HOLTFRETER (1944, 1945, 1946*a*, *b*, 1947, 1948*a*, *b*) on the behaviour of ectodermal cells disaggregated by injuroious agents could be confirmed by the present data. However a few minor discrepancies may be pointed out. HOLTFRETER (1948) stated that the faculty of a cylindrical stretching of disaggergated embryonic cells foreshadows a neural differentiation. It should be noticed however that the cells treated with ammonia have little tendency to elongate, but their aggregate differentiates neural tissues. Furthermore cells disaggregated by the "Ca-free" medium or by mechanical means do elongate, nevertheless they do not reveal any sign of neuralization.

HOLTFRETER (1945, 1947, 1948a) concluded that the closer the cells were brought to a state of irreversible cytolysis, the greater was their tendency to develop in neural direction. In the present results, however, the cell mass exposed to ammonia for a longer period was not able to differentiate neural tissue, suggesting that an optimum range of the stimulus exists for dorsalization.

SUMMARY

1. The isolated gastrula ectoderm of *Triturus pyrrhogaster* differentiated into the epidermis but not into neural tissues when disaggregated by "Ca-free" HOLTFRETER solution, M/8 sucrose, M/16 lithium chloride, or by 15% alcohol, reaggregated in HOLTFRETER solution and cultured. Thus disaggregation of the gastrula ectoderm is not the sufficient condition for dorsalization.

2. The ectoderm was brought to differentiate neural tissue and mesenchyme by a moderate treatment with ammonia (pH 12.0), sodium hydroxide (pH 12.0), hydrochloric acid (pH 2.0), urea (M/16), and sodium thiocyanate (M/32). Solation of the endoplasm and formation of pseudopodia were observed in cells immediately after the treatment with these agents.

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* Many of the animalizing agents for the echinoderm eggs are found to have dorsalizing effect on the amphibian eggs (cf. the following paper of this series).

3. A moderate exposure of the ectoderm to the "Ca-free" medium or to other non-dorsalizing solutions hardly caused such active protoplasmic movements.

4. A prolonged exposure to ammonia led the ectoderm to differentiate into the epidermis but not to neural tissues.

5. The possible role of solation of the endoplasm, brought about by ammonia etc., in the process of dorsalization was suggested.

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EMBRYOLOGIA, Vol. 3, No. 4, pp. 335-341 (December, 1957)

ON THE MECHANISM OF THE DORSALIZATION IN THE ECTODERM OF *TRITURUS* GASTRULAE CAUSED BY PRECYTOLYTIC TREATMENTS

II. THE SUPPRESSION OF DORSALIZATION BY TREATMENTS WITH SUCROSE, GLUCOSE AND 2,4-DINITROPHENOL

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INTRODUCTION

In the previous paper of this series, it has been shown that some agents (ammonia, sodium hydroxide, hydrochloric acid, urea, and sodium thiocyanate) with a dorsalizing effect on the ectoderm of *Triturus* gastrula cause active protoplasmic movements in the separated cells immediately after treatments, and that such protoplasmic change is slight when non-dorsalizing agents ("Ca-free", sucrose, and lithium chloride) are applied on the same ectoderm. In order to test the possibility that the changes of protoplasm observed immediately after exposure to the dorsalizing agents are causally connected with their effects on the subsequent differentiation of the cells, the ectoderm was first exposed to the dorsalizing agents and then to sucrose, glucose or 2,4-dinitrophenol (DNP), which were found to have a tendency to suppress dorsalization. It was hoped that a study of the state of protoplasm immediately after the combined exposure might give a clue for understanding the mechanism controlling the differentiation of embryonic cells.

MATERIAL AND METHODS

Some of the solutions used in the present experiment were prepared as follows:

Sucrose-HOLTFRETER solution: M/8 sucrose and HOLTFRETER solution were mixed in the ratio of 1 : 1 or 1 : 3, and buffered at pH 7.0.

Glucose-HOLTFERTER solution: M/8 glucose diluted with the same volume of HOLTFRETER solution and buffered at pH 7.0.

DNP-HOLTFRETER solution: HOLTFRETER solution containing 5×10^{-5} M or 10^{-4} M 2, 4-dinitrophenol buffered at pH 7.0.

For other details, see the first paper of this series.

RESULTS

I. Effects of sucrose or glucose subsequent to ammonia treatment

After an exposure to ammonia for 2–5 minutes, the ectoderm was transferred to sucrose-HOLTFRETER solution. Observed under the phase contrast microscope, free cells did not show the active protoplasmic movements, which were usually observable after treatment with ammonia. The area of ectoplasmic layer was decreased and the endoplasmic layer appeared solidified (Fig. 1 and 2). A very conspicuous suppression of protoplasmic movements was observed when cells were transferred to a pure M/8 sucrose solution after the exposure to ammonia. In this medium cells were shrunken, the cytoplasm appeared coagulated, and hyaline lobopodia disappeared. In solutions containing sucrose, reaggregation proceeded more rapidly and more thoroughly than in HOLTFRETER solution.

Table 1. Effect of sucrose on the differentiation of the ammonia-treated ectoderm (Series I)

Series	Concentration of sucrose	Solation of endoplasm	Duration of exposure	Total number available	Dorsalized	Non-dorsalized	Histological constitution of explants				
							Brain	Neural mass	Neuroid*	Mesenchyme	Melanophore
Ia	M/32	±	9 days	14	3	11	0	0	3	0	0
Ib	M/16	±±	24 hrs	35	2	33	0	0	2	0	0
Ic	M/16	±	9 days	27	1	26	0	0	1	0	1
Ammonia alone**		##		15	12	3	2	6	4	1	1
Sucrose alone (M/16 or M/32)		–	9 days	8	0	8	0	0	0	0	0

* Cell condensation without a clear neural character.

** Treatment with ammonia for the same duration as in series Ia–Ic without subsequent exposure to sucrose.

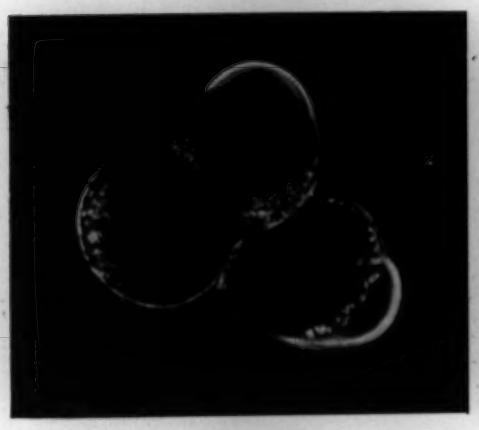


Fig. 1

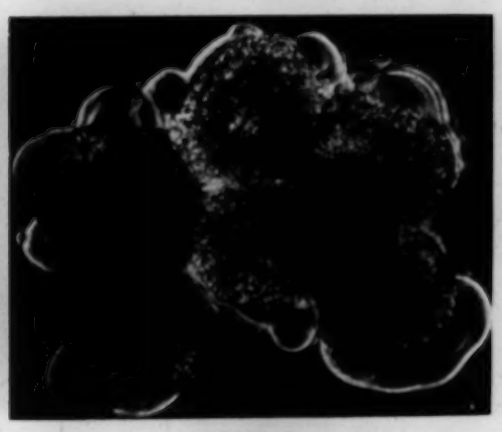


Fig. 2

Fig. 1. Embryonic cells which were transferred to M/16 sucrose-HOLTFRETER solution after the shock treatment with ammonia (Series I b). Dark-contrast. ×320.

Fig. 2. Embryonic cells which were transferred to HOLTFRETER solution after the shock treatment with ammonia (Series I, control). Dark-contrast. ×320.

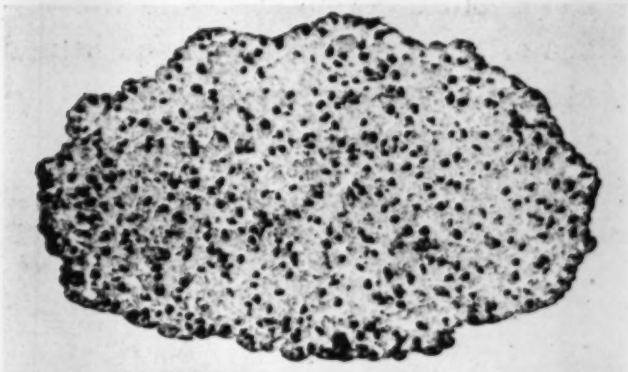


Fig. 3. An aggregate of epidermal cells, differentiated from the ectoderm cultured in M/16 sucrose-HOLTFRETER solution after ammonia treatment (Series I b). ×45.

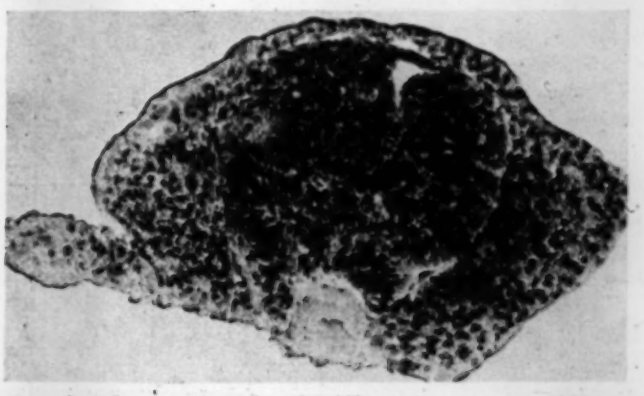


Fig. 4. An irregular brain-like structure differenitiated from the isolated ectoderm after exposure to ammonia solution (Series I, control). ×45.

In order to study the morphogenetic effects of sucrose, the ammonia-treated cells were either left in sucrose-HOLTFRETER solution for 24 hours and transferred to HOLTFRETER solution and cultured, or kept in sucrose-HOLTFRETER solution for the whole duration of culture. The dorsalization of the ectoderm evoked by ammonia was definitely suppressed in all experimental series (Table 1).

Two sets of control experiments were conducted. In the first control series, explants were exposed to ammonia for the same duration as in the experimental series, and then cultured in HOLTFRETER solution. Confirming the similar experiments reported in the first paper of this series, a high frequency of dorsalization was evidenced (Table 1 and Fig. 4).

In the second control series, the freshly isolated ectoderm was treated with M/16 or M/32 sucrose-HOLTFRETER solution. The isolated ectoderm survived in those solutions more than 8 days without any sign of disintegration, and differentiated into the atypical epidermis. Histological sections of these explants revealed little delay in development as compared with untreated one.

A similar set of experiments were carried out using glucose instead of sucrose (series II). As shown in Table 2, glucose inhibited protoplasmic movements as well as dorsalization evoked by ammonia treatment.

Table 2. Effect of glucose on the differentiation of the ammonia-treated ectoderm (Series II)

Series	Concentration of glucose	Solation of endoplasm	Duration of exposure	Total number available	Dorsalized	Non-dorsalized	Histological constitution of explants				
							Brain	Neural mass	Neuroid*	Mesenchyme	Melanophore
II	M/16	±	9 days	15	1	14	0	0	1	0	0
Ammonia alone**		##		17	11	6	2	7	2	0	0
Glucose alone (M/16)		–	9 days	7	0	7	0	0	0	0	0

* Cell condensation without a clear neural character.

** Treatment with ammonia for the same duration as in series II without subsequent exposure to glucose.

II. Effect of DNP subsequent to ammonia treatment

When transferred to DNP solution after 3–5 minutes exposure to ammonia, each cell began active protoplasmic movements sending out numerous cylindrical pseudopodia. The rapid succession of solation and gelation at the endoplasmic layer accompanied the movement of the ectoplasm. Thus no indication was obtained that DNP suppresses the active protoplasmic movements induced by ammonia treatment.

DNP did not affect the cellular adhesiveness, so that the cells dispersed by ammonia treatment reaggregated in DNP-HOLTFRETER solution. After 24 hours the reaggregates were transferred to HOLTFRETER solution, and cultured as usual. During the culture discharge of a large amount of free cells was observed, which caused noticeable reduction in the size of reaggregates. All the survived reaggregates differentiated into irregularly arranged epidermal cells. As is clear from Table 3, dorsalization was completely suppressed.

Table 3. Effect of 2, 4-dinitrophenol treatment on the differentiation of the ammonia-treated ectoderm (Series III)

Series	Concentration of DNP	Solation of the endoplasm	Duration of exposure	Total number of explants	Dorsalized explants	Non-dorsalized explants	Histological constitution of explants: Brain	Neural mass	Neuroid*	Mesenchyme	Melanophore
III a	5×10^{-5} M	##	24 hrs	27	0	27	0	0	0	0	0
III b	10^{-4} M	##	24 hrs	29	0	29	0	0	0	0	0
Ammonia alone**		##		18	11	7	3	4	4	1	1
DNP alone (5×10^{-5} M or 10^{-4}M)		–	24 hrs	15	0	15	0	0	0	0	0

* Cell condensation without a clear neural character.

** Treatment with ammonia for the same duration as in series III a–III b without subsequent exposure to DNP.

In the first control series, explants kept in HOLTFRETER solution after ammonia treatment differentiated to neural or neuroid tissue in 61% of cases (Table 3), confirming the results reported in the first paper.

The effect of DNP alone was studied in the second control series. The freshly isolated ectoderm was kept in 10^{-4} M DNP for 24 hours,

then transferred to and cultured in HOLTFRETER solution. On histological examination all explants showed good differentiation of epidermal cells.

DISCUSSION

The results obtained indicate that dorsalization of the ectoderm by an ammonia treatment is suppressed by subsequent treatment with sucrose or glucose. Cytological observations reported in the first paper have shown that ammonia has a liquefying effect on the endoplasm and induces active protoplasmic movements. The present experiments demonstrated that sucrose or glucose suppress these protoplasmic movements activated by ammonia. All these results can be interpreted by assuming that the ammonia-induced change in the protoplasm is the initial step of dorsalization, and that this change is suppressed by sucrose and glucose. HOLTFRETER (1947) found that the ectodermal explants of *Amblystoma punctatum* which differentiated in the neural direction in the standard solution could be forced to differentiate into the epidermal direction by adding glucose or sucrose. Although the procedure and species were different in his experiment from those of the present ones, the mechanism of the suppression of dorsalization probably has a common base in both cases.

In another experiment, lithium chloride has also been shown to have a tendency to suppress dorsalization of the ammonia-treated ectoderm (KARASAKI, unpublished). The ectodermal explants pretreated with ammonia was exposed to lithium chloride at a higher concentration (M/48 to M/32) for 24 hours, and then cultured in HOLTFRETER solution. The results indicated a slight suppression of dorsalization. OKANO (1957) obtained a better suppression of dorsalization, when he treated the ectoderm first with lithium chloride and then with lactic acid which was used as the dorsalizing agent. As lithium chloride is known to have a stabilizing effect on the cell structures (cf. RAVEN and VAN ZEIST, 1950; RANZI, 1952), it is rather probable that the substance operates in an essentially similar way to that assumed for sucrose.

Of the agents examined in this paper, DNP was found to be most effective in suppressing dorsalization of the ectoderm. However as

it hardly affects the state of protoplasm, the mode of suppression of dorsalization in this case should be different from that of sucrose or glucose. In view of the well-known effect of DNP on the phosphorylation associated with oxidation, it may affect directly the metabolic activity of the developing cells. It is not improbable that the protoplasmic change caused by ammonia leads to an enhancement of metabolic activities which are essential for dorsalization, and that DNP attacks this phase, leaving the protoplasmic changes unaltered. If this be the case, sucrose, glucose and lithium chloride affect the earlier phase and DNP the later phase of the chain of events which makes up dorsalization.

Summary

1. Dorsalization of the isolated ectoderm of *Triturus pyrrhogaster* induced by ammonia was suppressed by subsequent treatment with sucrose, glucose or 2, 4-dinitrophenol (DNP).

2. The treatment with sucrose or glucose inhibited the protoplasmic changes produced by ammonia in the ectodermal cells. On the other hand DNP was found ineffective in suppressing these changes.

3. Implications of these data were discussed together with some data of lithium-treatment.

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STUDIES OF THE METAMORPHOSIS IN INSECTS

II. RELATION BETWEEN PUPATION AND THE TRACHEAL SYSTEM IN *DROSOPHILA MELANOGASTER*

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INTRODUCTION

In the mature larva of the fruit fly, *Drosophila melanogaster*, the tracheal system opens to the outside air through the anterior and posterior spiracles. If the mature larva is ligated at two levels along the body axis 12 hours before the presumptive pupation time at 25 °C., subsequent pupation is restricted to the anterior and posterior part, and the middle part is retained in the larval condition (BODENSTEIN 1938, TAKAOKA 1951). The result can hardly be explained by a difference in the hormonal condition of different parts of the body, because according to BODENSTEIN a sufficient amount of the pupation hormone must be present throughout the larval body at the stage of the experiment. A similar suppression of pupation by constriction can be obtained also in the larva of the blowfly, *Calliphora erythrocephala* (FRAENKEL 1935).

In the case of *Sturmia sericariae*, the larval tracheal system is in continuation with the outside air only through the posterior spiracles. According to FUKUDA (1937), pupation can be reversibly prevented by blocking the posterior spiracles with gelatin. All these facts seem to suggest the possibility that in the presence of a sufficient amount of pupation hormone, pupation is dependent upon the air supplied by the tracheal system.

In order to test the idea, the tracheal system of the mature larva of *Drosophila melanogaster* was cut in various ways, and the effect of cutting on pupation was compared with that of the corresponding ligation experiments. In the ligated larva, the body fluid is divided into parts, while in the larva with the cut trachea the

body fluid is left circulating. Therefore such experiments may contribute to an understanding of the relative significance of the tracheal system and body fluid for pupation.

The writer wishes to express his sincere thanks to Prof. Isao Motomura of the Tôhoku University by whose suggestion this work was initiated, and also to Prof. Tuneo Yamada of the Nagoya University for his valuable criticism.

Material and Method

The material used was mature larvae of *Drosophila melanogaster*. Only the individuals which pupated within 7 hours after treatment at 25° ±0.5° C. were employed. As earlier experiments of Bodenstein (1938) showed that the larva of *Drosophila melanogaster* possesses pupation hormone in an amount sufficient for pupation at all levels of the body already 12 hours before pupation, it was assumed that the concentration of the hormone in the present material was adequate for pupation.

In order to cut the tracheal system or to ligate the larval body, the following three methods were adopted:

(*1*) *Ligature-removing method.* Severance of the lateral trunks and branches of the tracheal system was achieved by ligating the

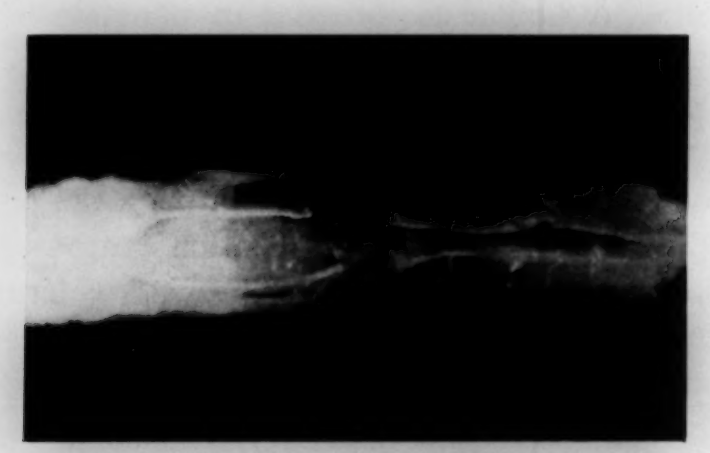


Fig. 1. A larva in which the lateral trunks and branches of the tracheal system were cut with ligature-removing method, showing cut ends of the lateral trunks of the tracheal system and the retraction of the cut ends. The anterior end was shifted forwards and the posterior end backwards.

larva with a hair loop, which was removed immediately afterwards. In this case, the tracheal tubes, fat bodies and Malpighian tubes were severed but the alimentary canal. Immediately after the operation, withdrawal of the cut ends of the tracheal tubes was observed: The anterior end was shifted forwards and the posterior end backwards leaving an area free of tracheal tubes (Fig. 1).

(*2*) *Nipping method.* The lateral trunks of the tracheal system were cut by nipping them with fine forceps from the outside of the skin. When this method was applied to the tracheal tubes at the base of the process of the caudal segment, the tracheal tubes could be cut completely without disturbing other organs. The results of the operations could be checked by direct observation from the outside.

(*3*) *Ligating method.* Larvae were ligated with a hair loop as in the earlier experiment.

The ligation in the abdomen were carried out in the 7th, 8th, or 9th segment. The posterior spiracles were severed at the base of the process of the caudal segment. The ligation at the anterior spiracles was carried out just behind them.

Schemes of different types of experiments reported in this paper and results obtained are summarized in Table 1.

Carmine method. Although in the living larva, the tracheal system is visible through the skin, its observation was facilitated by feeding the larva in advance with carmine. The white tracheal system was then sharply contrasted against the red background of the alimentary canal.

Experiments

A. Trachea-cutting experiments

Exp. I Trachea-cutting in the abdomen (Table 1, Fig. 2).

Exp. II Trachea-cuttiug at two levels in the abdomen (Table 1, Fig. 3).

The operation was carried out at the anterior and posterior abdomen respectively.



a *b*

Fig. 2. Trachea-cutting in the abdomen. (*a*) A narrow transverse zone along the cut did not pupate, or (*b*) the hind part alone pupated.



Fig. 3



Fig. 4

Fig. 3. Trachea-cutting at two levels in the abdomen. The middle part did not pupate.

Fig. 4. Ligating at two levels in the abdomen. The middle part did not pupate.

Exp. III Trachea-cutting in the abdomen and at the posterior spiracles (Table 1).

B. Ligation experiments

Exp. IV Ligating at the anterior spiracles (Table 1).

Exp. V Ligating at the posterior spiracles (Table 1).

Exp. VI Ligating in the abdomen (Table 1).

Exp. VII Ligating at two levels in the abdomen (Table 1, Fig. 4). By two ligatures at the anterior and posterior abdomen, three body parts were separated.

C. Combination experiments

Exp. VIII Ligating at the anterior spiracles and trachea-cutting in the abdomen (Table 1, Fig. 5).



Fig. 5

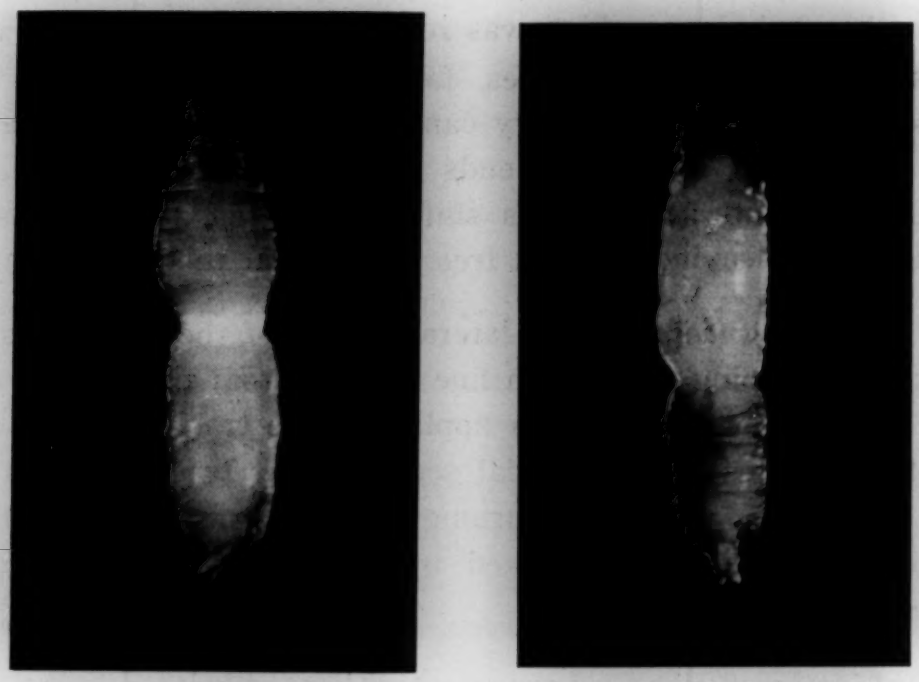


Fig. 6

Fig. 5. Ligating at the hinder part of the anterior spiracles with simultaneous cutting of the tracheal tubes in its abdomen. The posterior part alone pupated.

Fig. 6. Ligating at the base of the process of the caudal segment with simultaneous cutting of the tracheal tubes in the abdomen. The part anterior to the position of cutting pupated.

Exp. IX Trachea-cutting in the abdomen and ligating at the posterior spiracles (Table 1, Fig. 6).



a *b*

Fig. 2. Trachea-cutting in the abdomen. (*a*) A narrow transverse zone along the cut did not pupate, or (*b*) the hind part alone pupated.



Fig. 3



Fig. 4

Fig. 3. Trachea-cutting at two levels in the abdomen. The middle part did not pupate.

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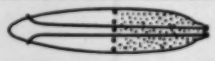
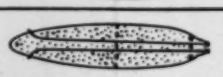
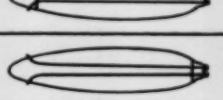
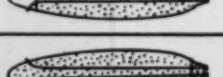
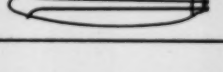
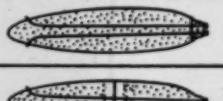
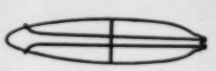
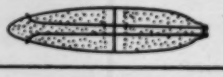
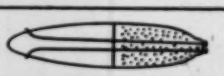
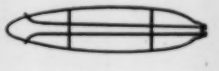
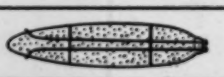
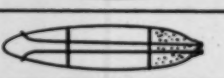
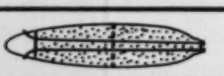
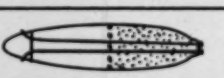
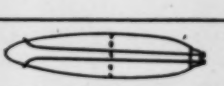
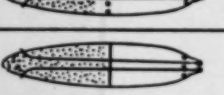
Fig. 6

Fig. 5. Ligating at the hinder part of the anterior spiracles with simultaneous cutting of the tracheal tubes in its abdomen. The posterior part alone pupated.

Fig. 6. Ligating at the base of the process of the caudal segment with simultaneous cutting of the tracheal tubes in the abdomen. The part anterior to the position of cutting pupated.

Exp. IX Trachea-cutting in the abdomen and ligating at the posterior spiracles (Table 1, Fig. 6).

Table 1. Schemes of different types of the cutting and ligating experiments of *Drosophila* larvae

	No. of exp.	Treatments	Results obtained		
				Nos.	Total
A Trachea-cutting experiments	I			72	84
				12	
	II			8	62
				40	
				14	
	III			1	31
				30	
B Ligation experiments	IV			30	30
	V			30	30
	VI			114	127
				13	
	VII			12	90
				56	
				22	
C Combination experiments	VIII			3	54
				51	
	IX			30	30
	X			30	30

 Position of cutting.

 Position of the ligature.

 Pupated part.

 Unpupated part.

Exp. X Ligating in the abdomen and trachea-cutting at the posterior spiracles (Table 1).

Results are diagrammatically shown in Table 1.

Discussion

As to the significance of the air supply for pupation, the following results appear pertinent: Subsequent to a cut of the tracheal tubes of mature larvae in the abdomen, pupation is observed in the anterior and posterior part in most of the larvae (Exp. I, Table 1, Fig. 2 *a*). However, when the tracheal tubes, *i.e.* the lateral trunks with associated branches, are cut at two levels in the abdomen, the middle part alone fails to pupate (Exp. II, Table 1, Fig. 3). Simultaneous disconnection of the lateral trunks in the abdomen and of those in the caudal segment was followed by pupation of the anterior part (Exp. III, Table 1). As the pupated part has the air supply by the spiracles, and the unpupated part is separated from the spiracles, one may conclude that in the mature larva of *Drosophila melanogaster* the tracheal communication with the spiracle is an important factor for the pupation of a given part of the larva. The conclusion is supported by the following group of results. When mature larvae are ligated into two parts, pupation is observed in both the anterior and posterior parts in most of the larvae presumably because the both parts are provided with spiracles (Exp. VI, Table 1). When they are ligated at two levels into three parts (Exp. VII, Table 1), the middle part devoid of spiracles does not pupate in most of the larvae and the anterior and posterior parts behave as in Exp. VI (Fig. 4). The corresponding trachea-cutting experiments (Exps. I and II) give the same results (Table 1). In the cutting experiment (Exps. I and II), circulation of the body fluid between the two parts is not interrupted, while in the ligation experiment (Exps. VI and VII), flow of the body fluid between the two parts is interrupted. In spite of the difference in the condition of circulation, the pattern of pupation turned out to be the same. Further if disconnection of the tracheal tubes in the abdomen is combined with ligation of the lateral trunks at the base of the process of the caudal segment, only the part anterior to the position of cutting pupates (Exp. IX). The outcome is the same even if

ligation and severance are reversed in position (Exp. X). All these facts again show that the important factor for pupation is not the body fluid, but the connection of the tissue with the atmosphere by the tracheal system under the present experimental conditions. This result fits in with the conclusion reached earlier by FRAENKEL (1935), FUKUDA (1937) and BODENSTEIN (1938).

However, in discussing the results also the stage of experiments cannot be neglected. When the tracheal system is cut or ligated into three parts about 1–2 hours before the presumptive pupation time, the middle part does pupate together with the anterior and posterior part, although the former is cut off from the outside air (Exps. II, III, VII and VIII). This fact seems to suggest one of the following alternatives: Either the middle part can continue to develop for a certain duration after the operation, consuming the oxygen contained in it and pupates, or about 2 hours before the beginning of pupation, the process is determined to occur and, if it is once determined, oxygen is not required for its realization.

When the trachea-cut larva pupates, the limit of the pupated part does not correspond exactly with the position of cutting (Figs. 2 *a*, 2 *b* and 5). This fact is easily explained from the observation that when the tracheal tubes are cut, their cut ends retract at once (Fig. 1). Consequently, the position of cutting does not exactly correspond to the position of the cut ends.

When the mature larva of *Drosophila melanogaster* is ligated into two parts at the critical period of secretion of the pupation hormone, pupation in the posterior part is rarely observed although the pupation hormone is assumed to be already present in the anterior part (BODENSTEIN 1938, TAKAOKA 1951).

The same phenomenon was also observed by FRAENKEL (1935) in the blowfly, *Calliphora erythrocephala*. According to TAKAOKA (1951), the frequency of these cases in *Drosophila melanogaster* has no relation with the position of the ligature. FRAENKEL (1935) and BODENSTEIN (1938) have ascribed the suppression of pupation to lack of oxygen in the anterior part.

How is the supply of oxygen to the anterior part interrupted? A similar result also appears in the experiments with two ligatures

(Exp. VII) and in those of trachea-cutting (Exp. I, Fig. 2 *b*, Exp. II): Pupation is limited to the posterior part. The observed failure of pupation may be due to defects in the anterior tracheal system, especially in anterior spiracles, atria, or anterior part of lateral trunks.

Summary

1) The role of the tracheal system for pupation of mature larvae of *Drosophila melanogaster* was studied by cutting tracheal tubes with the ligature-removing method and nipping method, and by comparing these results with those of the corresponding ligation experiments.

2) When the lateral trunks and branches of the tracheal system are cut in the abdominal part of mature larvae, in some cases both the anterior and posterior parts pupate and in other cases only the posterior part pupates.

3) Disconnection of the lateral trunks and branches of the tracheal system at two levels along the body axis in the abdomen about 2–7 hours before the presumptive pupation time is followed by failure of pupation in the middle part.

4) If the tracheal tubes are cut at one level of the body axis and one end of the tubes is in contact with the outside air through the spiracles, pupation occurs in the region supplied with oxygen by the spiracles.

5) The trachea-cutting experiments which leave the body fluid circulation unaltered give the same results as those obtained by the comparable ligation experiments. Hence the circulatory condition cannot be an important factor for the pattern of pupation. Some of the well-known results of ligating experiments of mature larvae could be ascribed to disconnection of the tracheal system caused by ligating.

6) When cutting or ligating the tracheal system into three parts is done as late as about 1–2 hours before the presumptive pupation time, larvae can pupate completely, even if the middle part of the body is cut off from the air supply.

7) The individuals in which the posterior part alone pupates are occasionally observed in the trachea-cutting experiment, as well as

in the ligation experiment. An obstruction in the anterior spiracles, atria or anterior part of lateral trunks is suggested to be a possible cause for the failure of pupation in the anterior part.

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THE EMBRYONIC INDUCTIVE ACTION OF NORMAL AND REGENERATING RAT LIVER

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In the rat, partial hepatectomy is followed by intensive regeneration of the liver, which in a short time leads to restoration of the normal state. The rapidly growing tissue so obtained was used as implantation material in induction experiments for the following reasons. Firstly, the assumption advanced by LEVANDER (1949, 1956), among others, that the same substances which regulate embryonic differentiation also regulate later growth and regeneration led us to anticipate that differences in inductivity might be revealed by comparative studies on the same tissue during intensive growth and during rest. Secondly, such a comparison seemed useful in view of the fact that differences in inductive effect had previously been observed between normal tissue and the corresponding, rapidly growing malignant tissue (SAXÉN and TOIVONEN 1956, TOIVONEN and SAXÉN 1957). And thirdly, it is known that the amount of nucleic acids is markedly increased in regenerating liver (see discussion). Hence it seemed possible that the controversial topic of the role of the nucleic acids in the induction process might be elucidated by comparison of this tissue with normal liver tissue.

MATERIAL AND METHODS

On adult male Wistar rats weighing about 200 g. partial hepatectomy, involving the removal of 60–70 per cent of the liver, was performed under ether anaesthesia by HIGGINS and ANDERSON's (1931) technique. The rats were killed 72 hours later. Normal individuals of the same size were used as controls. Part of the liver was fixed and stained for histological investigation, and the mitoses were counted in 300 fields (Bouin, haemalum-eosin, obj. 40 x, oc. 10 x). The results obtained from the various preparations are shown in Table

1. In three normal livers similarly studied a total of no more than two mitoses was encountered.

Table 1. Number of mitotic figures per 100 visual fields in the regenerating liver of the rat 72 hours after partial hepatectomy

		Counts			Mean
		1	2	3	
Rat	N:o 1.	41	24	24	30
〃	〃 2.	9	16	13	13
〃	〃 3.	14	21	31	22
〃	〃 4.	1	1	1	1

Since reliable data are available on the nucleic acid concentration in regenerating liver tissue, the livers employed in the implantation experiments were not tested in this respect. Table 2, however, shows the results obtained in our laboratory on a regeneration series in which the rats employed were of the same strain and reared under the same conditions as those used in the present investigation.

Table 2. Amount of DNA and RNA in regenerating liver after partial hepatectomy (per cent of dry weight) (TEIR et alii, unpubl.)

		DNA	RNA
Control	(16 rats)	1.13	3.71
24 hours after oper.	(4 〃)	1.46	4.41
48 〃 〃 〃	(6 〃)	1.36	4.90
96 〃 〃 〃	(6 〃)	1.50	5.58

The liver tissue to be used for implantation was minced, and fixed for 8–24 hours in 70 per cent alcohol, after which it was implanted in classical fashion into *Triturus* gastrulae. The embryological methods employed have previously been described in detail (*e.g.* TOIVONEN and SAXÉN 1955).

RESULTS

In the control experiments the livers of three rats were used, and a total of 95 cases were available. The regenerating liver tissue

derived from four individuals was tested by implantation. In this series 97 cases were available. The results are shown in Tables 3, 4 and Diagram 1.

The liver implants proved to be relatively weak and purely archencephalic inductors. On comparing the induced structures in the

Table 3. Inductive action of normal and regenerating liver

Donor	Number of cases		Induction	Induced structures				
	Total	Positive	(percentage)	Forebrain	Eye	Nose	Balancer	Neural cells
				(percentage and number of cases)				
Control								
1	27	17	60	41(11)	22(6)	30(8)	18(5)	15(4)
2	34	28	82	44(15)	30(10)	21(7)	35(12)	21(7)
3	34	23	68	41(14)	27(9)	27(9)	21(7)	15(5)
Total	95	68	74	42(40)	26(25)	25(24)	24(23)	17(16)
Regenerating								
1	11	8	73	45(5)	36(4)	18(2)	27(3)	18(2)
2	28	26	89	53(15)	46(13)	32(9)	22(6)	32(9)
3	27	13	48	22(6)	15(4)	18(5)	18(5)	15(4)
4	33	22	66	39(13)	24(8)	21(7)	33(11)	9(3)
Total	97	69	71	40(39)	30(29)	24(23)	26(25)	19(18)

Table 4. Percentage distribution of the induced structures in the positive cases in liver implantation series

	Percentage				
	Forebrain	Eye	Nose	Balancer	Neural cells
Control					
1	65	35	47	30	24
2	53	36	25	45	25
3	61	39	39	30	22
Total	60	37	35	33	23
Regenerating					
1	62	50	25	37	25
2	58	50	53	23	35
3	46	30	38	38	30
4	59	36	30	50	14
Total	58	42	33	36	26

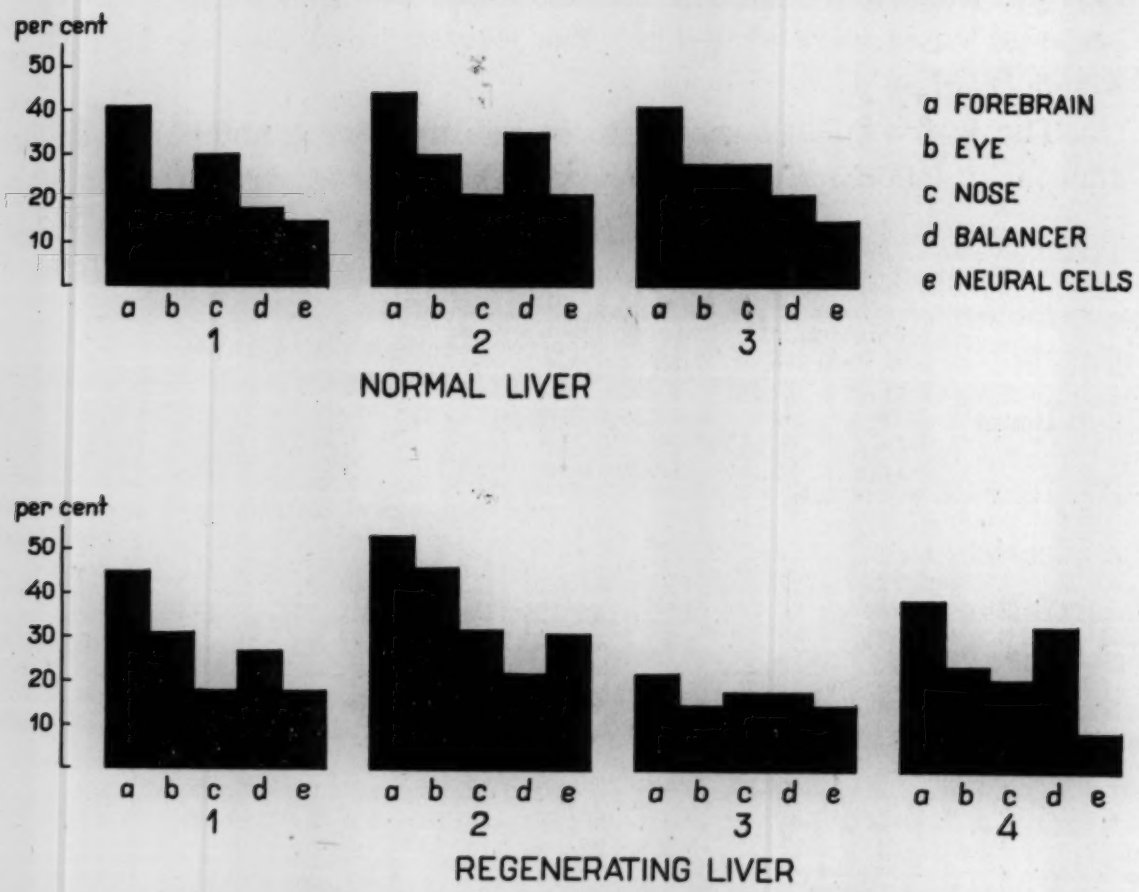


Fig. 1. Diagram showing the percentages of induced structures in implantation series with normal and regenerating liver of the rat.

two series, no differences were observable either qualitatively or quantitatively. In the hepatectomy series there were slight individual variations but, all cases considered, the two series were strikingly similar.

Discussion

The present results afford no corroboration of the theory referred to above that embryonic differentiation and later growth are similarly regulated. At least in this case, the greatly intensified growth and the concomitant changes in the chemical composition of the tissue (cf. Tsuboi, Yokoyama, Stowell and Wilson 1954) did not influence the neuralizing capacity of the latter. It has previously been observed, however, that the archencephalic inductive capacity of the liver, which in the present experiments proved to be stable, clearly changes under the influence of environmental factors, in particular

hunger. In implantation experiments TOIVONEN (1951) used guinea-pig liver from a normally fed individual and from an individual which had been starved for some days. Definite differences appeared in the results: The normal tissue induced both archencephalic and deuterencephalic structures, but fasting changed the reaction to an almost purely archencephalic one, and the number of archencephalic formations clearly rose. Today, however, we are inclined to believe that the influence on the archencephalic induction was only indirect. According to the gradient theories previously advanced (LEHMANN 1950, TOIVONEN and SAXÉN 1955), two groups of factors are active in the process of induction, one producing archencephalic differentiation alone, and another mesodermalization. Other types of induction are attributed to the combined influence of these two factors. Thus a certain neural/mesodermal ratio brings about deuterencephalic induction. On this interpretation, no change in the archencephalic (or neural) factor occurred in TOIVONEN's (op. cit.) experiments with liver, but the mesodermal factor was weakened, and this brought about cranialization of the neural induction. The change in the equilibrium would have been the same, had the neural factor become stronger and the mesodermal factor remained the same, but the interpretation rendered here is corroborated by both the present results and those of ENGLÄNDER and JOHNEN (1957) with organs from starved guinea-pig. These investigators repeated the hunger experiment with guinea-pig liver, which, however, differed from that used by Toivonen inasmuch as its normal effect was purely archencephalic. It was found that hunger had no effect. By contrast, when tissues were used which originally had a combined effect (deuterencephalic or spinocaudal), a change towards a more archencephalic reaction occurred, as in TOIVONEN's (op. cit) experiments. These results, as well as those obtained in the present regeneration series, seem to indicate that the archencephalic inductive capacity of the tissues is very stable, whilst the mesodermal factor is obviously much more labile.

Since BRACHET (1942, 1943) advocated the view that neural induction is closely associated with the nucleic acids, numerous investigators have studied this point, and KUUSI (1951) was the first to advance a contradictory opinion. This question has remained open, however, and the present study is an attempt to contribute to its

solution on the basis of new material. It has been shown in a large number of previous investigations that the nucleic acid concentration of the regenerating liver increases in relation to dry weight, protein nitrogen and cell count (PRICE and LAIRD 1950, NOVIKOFF and POTTER 1948, LESLIE 1955 and others). Determinations performed in our laboratory yielded the same result (Table 2). This increase is particularly marked in regard to RNA, and if RNA were immediately effective in neural induction it would seem logical to expect an intensification of the inductive capacity of the regenerating liver tissue as compared with the normal. In the present series the inductive capacity of the liver proved to be rather weak. More than one-fourth of the cases were completely negative, and definite cephalic structures were present in only 40 per cent. It is obvious that when negative cases and weak inductions occur, there are many contributory causes such as the area of the contact surface (and the size of the implant), the age and competence of the reactive ectoderm, etc. These, mainly technical, factors vary, however, within more or less the same limits in different experimental series, but apart from them, different tissues exhibit highly different, "organ-specific" inductive capacities. The rat bone marrow, for instance, is an almost 100 per cent inductor (SAXÉN and TOIVONEN 1956, TOIVONEN AND SAXÉN 1957), whilst the induction percentage of the guinea-pig liver is about 60, that of the guinea-pig kidney and parotid gland about 95, and that of the heart muscle of the same animal about 70 (ENGLÄNDER and JOHNEN 1957). The corresponding figure for the pigment epithelium of the frog's retina is less than 10 (TOIVONEN and SAXÉN, unpubl.). It seems highly probable that these differences are due in the first place to differences in concentration of the active substances in different organs. In the liver, for instance, this concentration is assumed to be so low that, for the tissue to bring about induction, more favourable conditions are required than, for instance, in experiments in which bone marrow, containing a larger amount of active factor, is used as implant. Consequently, an increase in the concentration of inductive factor in the tissue to be implanted should increase the inductive capacity of the latter. This view was also held by BRACHET (1947), who found that there is a clear correlation between the concentration of RNA in the tissues and tissue fractions and their inductive capacity, a finding which he regarded as corroboration of his

own theory regarding the close relationship between RNA and neuralization. If this theory were correct, it would be logical to assume, however, that an increase of the RNA concentration in the liver by almost 50 per cent would cause a change in the inductive capacity of the latter. The present results, which showed that the inductive capacity of normal and regenerating liver is both qualitatively and quantitatively similar, seem, however, strongly to indicate that RNA is, at least, not directly associated with the process of induction.

Summary

(1) Normal and regenerating rat liver was used as induction material in implantation experiments. The two series were both qualitatively and quantitatively similar in regard to their purely archencephalic inductive effect.

(2) The results seem to indicate that the neural factor in the tissues is stable and independent of external factors. Since there was a marked difference in RNA concentration between the two kinds of tissue employed, the writers conclude that no corroboration is obtained for the theory that ribonucleic acid plays a part in neuralization.

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THE THICKNESS OF THE CORTEX AND THE REFRACTIVE INDEX OF THE PROTOPLASM IN SEA URCHIN EGGS

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It has been believed that there is a cortex at the surface of the animal cell different both in morphological and in physical properties from the cell interior. The physiological importance of this part has been emphasized by many biologists (cf. JUST 1939, MITCHISON 1952, HEILBRUNN 1956). Although the presence of the cortex having definite physical properties has been shown by several investigators (cf. HEILBRUNN 1956), reports on the reliable measure of its thickness are scanty. In most of previous investigations, the presence of the cortex was ascertained by the fact that the distribution of the granules in the peripheral region of the cell was hardly affected by centrifugation while endoplasmic materials were easily stratified. Although it is conceivable that this cortical layer has a viscosity higher than that of the endoplasm, some doubts are left about exact coincidence of the extent of the layer marked by the granules not shifted by centrifugation with that of the cortex. Therefore, it is desirable to determine the exact position of the boundary between the gelated cortex and the fluid endoplasm by more direct methods. This work was done in order to get a reliable measure of the thickness of the cortex in sea urchin eggs by micromanipulation. In the calculation of the thickness, the correction of errors resulting from the refraction of light at the cell surface was made, to which previous investigators have paid no attention. This work also concerns with the determination of the refractive index of the egg protoplasm.

MATERIAL

The material used was the eggs of the sea urchin, *Hemicentrotus*

pulcherrimus. Before experimentation, the jelly of unfertilized eggs was removed by acid following DAN's procedure (1947), the fertilization membranes were removed by sucking the eggs into a fine pipette shortly after fertilization, and the hyaline layers were removed by immersion in calcium-free artificial sea water. The measurements were made in normal artificial sea water in unfertilized eggs and in calcium-free artificial sea water in fertilized eggs.

METHODS AND RESULTS

I. The refractive index of the protoplasm.

The refractive index of the egg was determined in the following way.

(*1*) An Indian ink mark was put on a slide. First, the image of the mark was focused through a microscope; secondly, an egg was inserted between the mark and the objective; the mark became out of focus owing to the lens-like effect of the inserted egg cell, so that it was required to move the tube of the microscope downwards for re-focusing on the image of the mark (cf. Fig. 1). The relation between the deviation of the image of the mark (x) and refractive index of the egg having a radius of unity is indicated in Fig. 2. Substituting x divided by the radius of the egg for x in Fig. 2, refractive index could be obtained from this graph.

As shown in Fig. 1, rays passing the mark on the slide are refracted at the cell surface and at the sea water-air interface.* Among these rays, only rays forming angles smaller than 40.54° ($\sin 40.54° = 0.65$) with the optical axis (in air) are used by the objective lens (N.A.: 0.65). The position of the image seen through the microscope is the arithmetic mean (p) of the position of the image made by paraxial rays (p_p) and that of the image made by marginal rays (p_m), which are determined by means of the trigonometrical ray tracing.

In the ray tracing, the value of the refractive index of the medium is

* Actually, the rays are refracted at the cell surface, the interface between sea water and the cover slip and the interface between the cover slip and air. However, the optical condition is considered to be equivalent to the condition in which the interface between the sea water and the cover slip is replaced by the sea water-air interface, since the objective lens is designed without aberration under the use of a cover slip of the standard thickness (0.17 mm.).

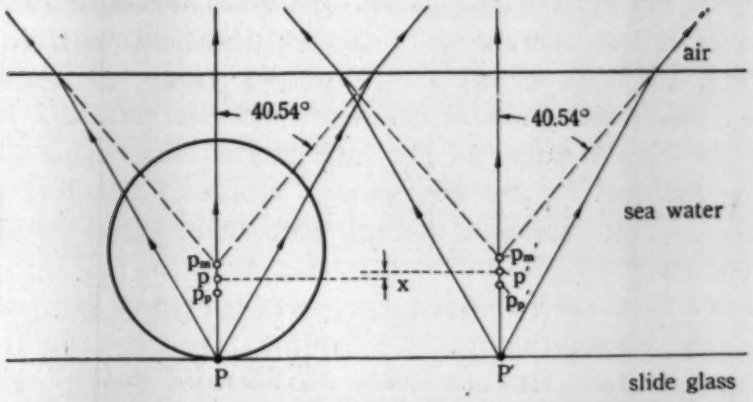


Fig. 1. Diagram indicating the principle of the determination of the refractive index of sea urchin eggs (the first method).

Left. Optical path passing a mark on the slide (P) through an egg placed over the mark. p_p: image of P made by paraxial rays. p_m: image of P made by marginal rays (rays forming the largest angle with the optical axis used by the objective lens). p: image of P seen through the microscope (mean position of p_p and p_m).

Right. Optical path passing a mark on the slide (P') through sea water. p_p' : image of P' made by paraxial rays. p_m': image of P' made by marginal rays. p': image of P' seen through the microscope (mean position of p_p' and p_m').

x: difference in positions of the above images (p, p'). For further explanation, see text.

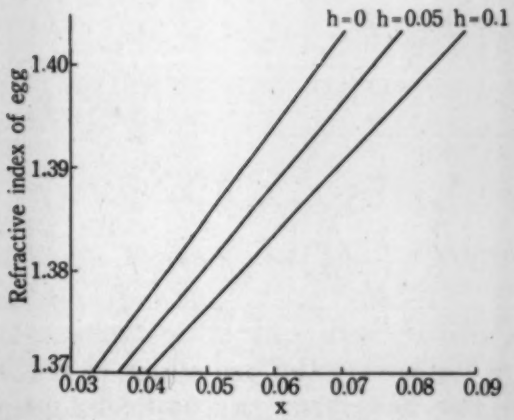


Fig. 2. Relations between refractive index of egg protoplasm and x values indicated in Fig. 1 and Fig. 3 *b*.

Curves indicate relations between refractive index and x for three values of h (thickness of the halo-layer). The radius of the egg is 1.00. Refractive index of the medium is 1.339.

necessary. The refractive index of artificial sea water or calcium-free artificial sea water is considered to be almost identical with that of isotonic (5/9 M) sodium chloride solution. Hence, a value of 1.3390 (15° C.) was obtained from tables for physical chemistry (BÖRNSTEIN and ROTH 1912).

The x is the displacement of the tube of the microscope for re-focusing, which was determined by the micrometer screw of the fine adjustment of the microscope. The radius of the egg was determined by means of a micrometer eye-piece.

On the surface of the fertilized egg, numerous fine processes are recognizable after the dissolution of the hyaline layer by the treatment with calcium-free sea water (HERBST 1900, MOTOMURA 1941). Because of the presence of this surface layer, which was called the halo-layer by MOTOMURA (1941), the cell surface is not directly in contact with the bottom of the vessel (HIRAMOTO 1955). The refractive index of the halo-layer was approximately the same as that of sea water, because the position of the image of the tip of a microneedle was not changed when an egg was brought close to the tip so as to make the tip come within the halo-layer (Fig. 3 *a*). In the case of the fertilized egg (Fig. 3 *b*), relations between the x and the refractive index of the egg are indicated in Fig. 2 ($h=0.05$, $h=0.1$), which were obtained by the ray tracing.

(*2*) The second method was as follows. The egg was compressed under the cover slip (Fig. 3 *c*). When a mark on a slide was observed through a microscope, its image appeared in different levels when the light passed through the egg protoplasm and when the light passed through the medium (artificial sea water or isotonic (0.75 M) sucrose solution*). The refractive index of the egg was obtained from the ratio x/D.

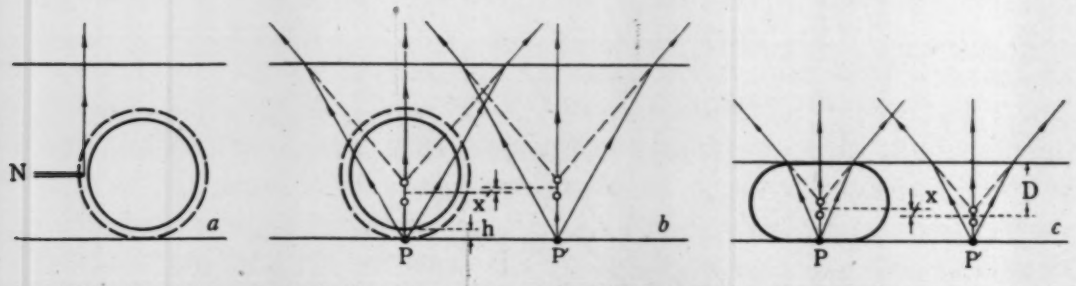


Fig. 3. Diagram indicating the principles of the determination of the refractive index of the halo-layer (*a*) and that of the egg protoplasm (*b*, *c*).

The surface of the egg and that of the halo-layer are indicated by heavy solid line and broken line, respectively. Paraxial ray in *a* and paraxial rays and marginal rays in *b* and *c* are indicated by arrows. *N*: microneedle inserted into the halo-layer. *P*, *P'*: marks on the slide. *h*: thickness of the halo-layer. For further explanation, see text.

* Refractive index: 1.3700 (15° C.) (BÖRNSTEIN and ROTH 1912).

The values of D and x illustrated in Fig. 3 *c* were determined by the micrometer scale of the fine adjustment of the microscope. Images of marks (P, P') on the slide, which could be determined by the ray tracing, are in mean positions of the positions of images by paraxial rays and those by marginal rays.

(*3*) The methods mentioned above are satisfactory for the determination of the refractive index of the egg protoplasm as a whole, but some possibilities are left that the refractive index may be different between the cortical regions and the endoplasmic ones.

A method was designed for the determination of the refractive index of the cortex. An egg was put into a narrow wedge-shaped manipulation chamber designed by the author (HIRAMOTO 1956). The chamber was made sufficiently thin so as to place the material within the focal length of the ordinary microscope condenser. A microneedle was deeply introduced into the egg so that the apparent

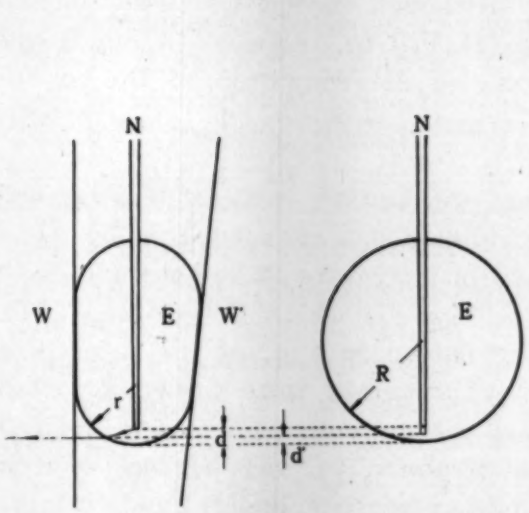


Fig. 4

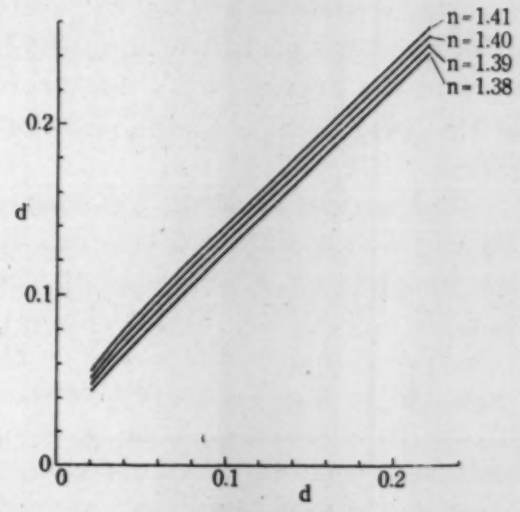


Fig. 5

Fig. 4. Optical path through the tip of a microneedle inserted into an egg in manipulation chamber.

The right figure indicates the view from the optical axis and the left figure the side view. E: egg. N: microneedle. R: major radius of curvature at the surface of the egg. r: minor radius of curvature at the surface of the egg. W, W': walls of the manipulation chamber.

Fig. 5. Relations between apparent distance of a point within the egg from the cell surface (d', cf. Fig. 4) and true one (d).

Curves indicate relations between d and d' for four values of the refractive index of the egg protoplasm ($n=1.380$, $n=1.390$, $n=1.400$, $n=1.410$). The refractive index of the medium is 1.339. The radius of curvature of the egg is 1.00.

distance between the tip of the needle and the cell surface (d') was about 2 μ (Fig. 4). After the position of the image of the needle tip within the egg was determined, the egg was removed while the position of the needle was kept fixed, and then position of the needle tip without the egg was determined. The light passing the needle tip is refracted at the cell surface, and therefore, the apparent distance between the needle tip and the cell surface (d' in Fig. 4) is different from the true distance (d). The deviation of the apparent distance from the true one depends upon the refractive index of the egg (chiefly of the cortical region of the egg) and the curvature of the egg surface. The relations between the apparent (d') and the true distance (d) for various values of the refractive index are indicated in Fig. 5. Using this graph, the refractive index of the cortex can be obtained.

The curves in Fig. 5 were obtained on the assumption that only paraxial rays were used by the optical system. Although rays other than paraxial ones also took part in making the image (cf. Fig. 6), relations in Fig. 5 were used in the present work because the precise determination of the position of the image was a complicated problem owing to the large spherical aberration.

The curvature of the cell surface was determined in the following way. An egg was compressed between two parallel plates manoeuvered by micromanipulators (P, P' in Fig. 7 *a*), and radii of curvature at the surface of the egg (R and r) and the thickness of the egg (D) were determined. The relations of r/r_0 to $D/2\,r_0$ and to R/r_0 are shown in Fig. 7 *b* for unfertilized eggs and in Fig. 7 *c* for fertilized eggs. The curves were drawn according to the data obtained from 6 unfertilized eggs in sea water and from 13 fertilized eggs in calcium-free sea water under various degrees of compression. From these curves, radius of curvature r could be determined from R and r_0, assuming that the walls of the chamber were parallel with each other.

The results of the determination of the refractive index are shown in Table 1. No change in the index of the fertilized egg during development could be detected. It will be seen that the refractive index of the cortical cytoplasm is not so much different from that of the egg protoplasm as a whole. The values of the refractive index obtained in the present study almost coincide with the values reported in eggs of other species of sea urchins (Vlès 1921 a, b, Mitchison and Swann 1953).

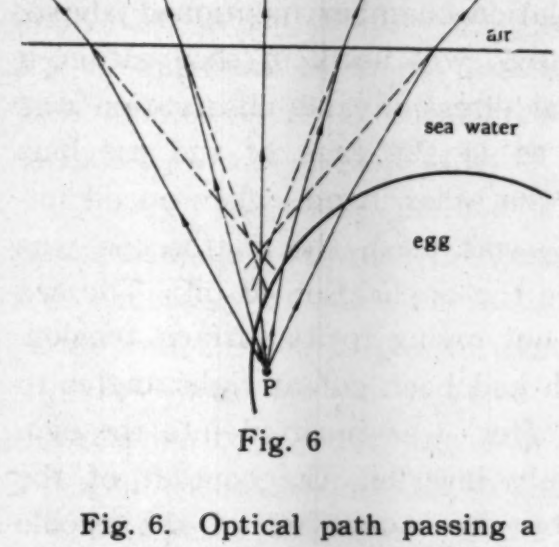


Fig. 6

Fig. 6. Optical path passing a point within a sea urchin egg.

Rays passing a point within the egg (P) are refracted at the cell surface and at the sea water-air interface. Consequently, the distance between the point and the cell surface appears smaller than true one. Five representative rays forming angles +40°, +20°, 0°, −20° and −40° with the optical axis of the microscope are indicated.

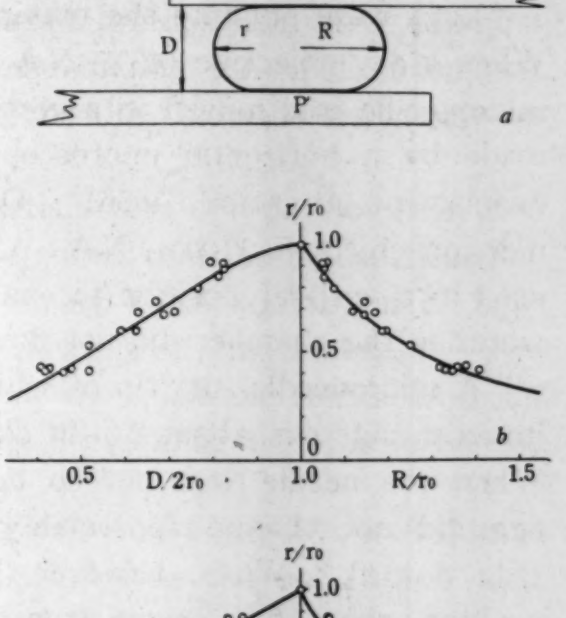


Fig. 7

Fig. 7. Relations among major and minor radii of curvature at the surface of the egg and the thickness of the egg when it is compressed between two parallel planes.

a. Diagram of a compressed egg. P, P': glass plate. D: thickness of the egg. R: major radius of curvature. r: minor radius of curvature.

b. $D/2\,r_0$ vs. r/r_0 (left) and R/r_0 vs. r/r_0 (right) in unfertilized eggs.*

c. $D/2\,r_0$ vs. r/r_0 (left) and R/r_0 vs. r/r_0 (right) in fertilized eggs.**

*, **: r_0 is the radius of the egg in uncompressed state.

Table 1. Refractive index of egg protoplasm (average)

	Medium	Method of determination		
		I	II	III
Unfertilized	Sea water	1.383	1.415	1.403
	Isotonic sucrose		1.379	
Fertilized	Ca-free sea water	1.376	1.406	1.406
	Isotonic sucrose		1.400	

II. The thickness of the cortex.

Eggs were put into the manipulation chamber mentioned above. When a dry objective (40×, N.A.: 0.65) was used for observation, a microneedle was moved in a vertical direction and observation was made by a horizontal microscope as in the case of the previous experiment (HIRAMOTO 1956). On the other hand, when an oil immersion objective (100×, N.A.: 1.25) was used, the microscope was used in a vertical position to enable the application of oil. The sea water in the chamber did not flow out owing to its surface tension.

A microneedle, the tip of which had been cut at right angles to its axis and was about 2 μ in diameter, was inserted into the egg. When the needle was not so deeply inserted, the contour of the egg did not change appreciably by the movement of the needle (Fig. 8 *a*, *b*). When, however, the tip of the needle reached the position about 2 μ away from the surface of the egg, the egg surface began to bulge out by further insertion (Fig. 8 *c*). This fact means that the tip had already been in contact with the gelated cortical layer. If the inserted needle was withdrawn, the surface of the egg was indented (Fig. 8 *d*), probably because of being pulled by the needle attached to the gelated layer. If the inserted needle was moved back and forth along its axis (Fig. 8 *e*) or was swung right and left within the egg (Fig. 8 *f*), the protoplasm around the needle was moved with it (arrows in Fig. 8 *e*, *f*), while the protoplasm within the peripheral zone of about 2 μ thickness was not

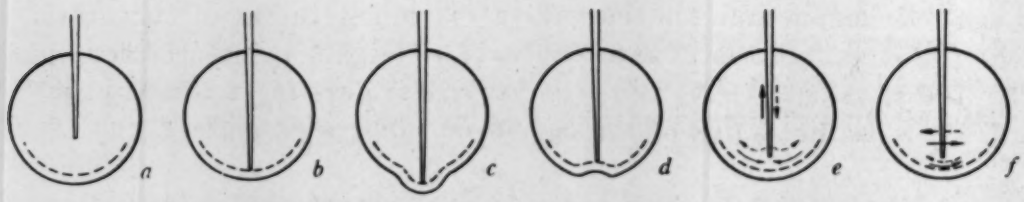


Fig. 8. Diagramatic representation of the methods of detection of the cortex in sea urchin eggs.

When the tip of a microneedle inserted into an egg is within the endoplasm, the contour of the egg scarcely changes by movement of the needle (*a*). After the tip reaches the cortex (*b*), the surface of the egg bulges out by further insertion of the needle (*c*), and it is indented by withdrawal of the needle (*d*). If the inserted needle is moved as shown by heavy arrows (*e*, *f*), the endoplasm moves together with the needle as shown by light arrows whereas the cortical cytoplasm does not.

moved by the movement of the inserted needle. These experiments imply that in sea urchin eggs a gelated protoplasmic layer of about 2 μ in apparent thickness exists surrounding the fluid endoplasm. This gelated layer is considered to be the "cortex" of the cell described by many investigators.

Though the above method is satisfactory for the determination of the boundary between the gelated cortex and the fluid endoplasm, it is necessary to make correction owing to the refraction of light at the cell surface to get an exact measure of the thickness. The true thickness can be obtained from the apparent thickness using the relation between d' and d in Fig. 6 assuming that the refractive index of cortical cytoplasm is 1.40.

Calculated thickness of the cortex are indicated in Table 2. The thickness of the cortex is about 3 μ in the unfertilized egg. The thickness of the cortex in the fertilized egg increases during development, until it reaches 4 μ shortly before cleavage. The thickness is almost the same over the entire surface of the egg in the above-mentioned stages. As furrowing begins, the thickness increases at the furrow region and decreases at the polar region. During interkinetic period, the thickness decreases at the furrow region and increases at the polar region. The results are somewhat schematically shown in Fig. 9.

In fertilized eggs of *Hemicentrotus pulcherrimus*, a row of orange-colored pigment granules is seen near the surface of the egg (cf. MOTOMURA 1935, 1941 b, DAN and DAN 1940, DAN 1954). According to MOTOMURA (1935), distribution of these pigment granules is not

Table 2. Thickness of the cortex in *Hemicentrotus* egg
(average values in μ)

Stage	Unfertilized (Fig. 9 *a*)	Early monaster (Fig. 9 *b*)	Late monaster (Fig. 9 *c*)	Amphiaster (Fig. 9 *d*)
	3.1	3.5	3.7	4.0

Stage	During cleavage		
	(Fig. 9 *e*)	(Fig. 9 *f-g*)	(Fig. 9 *h*)
Furrow region	4.3	4.3	3.1
Polar region	3.0	2.4	2.3

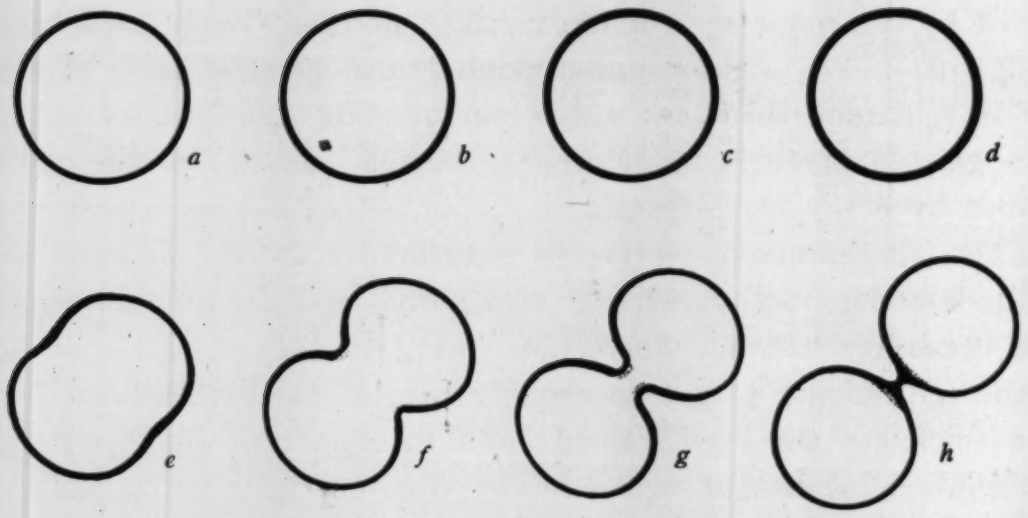


Fig. 9. Change in the thickness of the cortex in *Hemicentrotus* eggs at representative stages (180×).

a: unfertilized egg. *b*: fertilized egg in early monaster stage. *c*: late monaster stage. *d*: amphiaster stage. *e–h*: during cleavage. The layer of pigment granules in fertilized egg and cortex detected by micromanipulation coincide with each other in one cell stage (*b*, *c*, *d*) and in early stage of cleavage (*e*), but pigment granules are present also in the endoplasm as well as in the cortex at the furrow region of dividing egg in the late stage of cleavage while the pigment granular zone coincides with the cortex in other regions (*f*, *g*, *h*). Cortex is indicated by solid area and pigment granules dislodged into the endoplasm are indicated by black points.

affected by centrifugation, hence they are considered to be present in the cortex. In the present experiment, it was ascertained that the boundary between the cortex and the endoplasm exactly coincided with the inside of the row of pigment granules over the entire surface of the fertilized egg except the furrow region of the dividing egg. In dividing eggs, the pigment granules appeared to be dislodged into the endoplasm at the furrow region although they remained in the gelated cortex at the other regions (Fig. 9).

The thickness of the layer of pigment granules was practically unchanged when the egg was compressed between two parallel planes unless the degree of compression was too large. Therefore, it is concluded that the values mentioned above indicate the actual figures of the thickness of the cortex in uncompressed state, though the eggs were somewhat compressed in the manipulation chamber at the time of measurement.

Discussion

According to recent determination in centrifuged *Paracentrotus* and *Arbacia* eggs, the thickness of the cortex is 2 μ in living condition (Mitchison 1956). But in this determination, the correction of errors resulting from the refraction of light at the cell surface was not made. If the relation between r/R (the ratio of minor radius of curvature at the cell surface to major one) and $D/2r_0$ (the degree of compression) in *Hemicentrotus* eggs holds good for centrifuged eggs of *Paracentrotus* and *Arbacia*, it is possible to calculate the true thickness of the cortex in these eggs in the following way. According to figures in Mitchison's paper (Mitchison 1956, Fig. 1 *A*, *B*), R (major radius of curvature) is 48 μ in a *Paracentrotus* egg and 32 μ in an *Arbacia* egg, and D is 20 μ (Mitchison 1956, p. 111). If we look for the case in which $R/D=1.6$ (R/D value in *Arbacia*) is satisfied in Fig. 7 *b*, 0.40, 0.44 and 1.38 are obtained as r/r_0, $D/2r_0$ and R/r_0, respectively. Thus r in the centrifuged *Arbacia* egg is $r=D(r/r_0)/2(D/2r_0)=20\times0.40/2\times0.44=9.1$ (μ). By the same procedure, 8.8 μ is obtained as r in the *Paracentrotus* egg. Using the graph in Fig. 5, thickness of the cortex in centrifuged eggs of *Arbacia* and *Paracentrotus* is estimated to be 2.3 μ. In Mitchison's experiment (1956), surfaces of eggs were stretched considerably by centrifuging as well as by compression by cover slip. It was found by author's experiment that the surface area became about 1.4 times the original area when eggs were compressed between two parallel planes to a degree of 0.38 times the original diameter, which was the same order as the degree of compression in centrifuged eggs in Mitchison's experiment (1956) ($D/2r_0=0.44$ in the *Arbacia* egg, $D/2r_0=0.33$ in the *Paracentrotus* egg). The surface area of eggs in his experiment must be 1.45–1.5 times the original surface area in spherical state, if the elongation of the cell by centrifugation is taken into consideration. Assuming the constancy of the volume of the cortex under stretch, a value of 3.3–3.5 μ is obtained for the thickness of the cortex in the spherical egg. This value is quite analogous with that of *Hemicentrotus* eggs.

According to Motomura (personal communication), the thickness of the cortex in the *Hemicentrotus* egg is 5 μ in fixed material.

Chambers (1938) reported that an injected oil drop was indented

4–5 μ ahead of the advancing furrow in the egg of *Lytechinus*. If correction of errors owing to the refraction of light at the cell surface is made, a value of 5–6 μ is obtained for the true thickness of the cortex, which is not so much different from the thickness at the furrow region of the dividing *Hemicentrotus* egg. In an experiment similar to CHAMBERS' one (1938) which was attempted in *Hemicentrotus* eggs by the author, the injected oil drop (paraffin oil or olive oil) was not indented by the advancing furrow but slipped into one of the blastomeres during furrowing. Similar observations were also reported by CHAMBERS in *Arbacia* eggs (1938).

Whether the volume of the cortex changes or not is important in connection with the problem of new formation of the cell membrane during cleavage (cf. DAN 1954). The volume of the cortex must be unchanged when it is passively stretched or it contracts or expands actively, if no exchange of materials takes place between the cortex and the endoplasm. In other words, the change in the volume of the cortex implies the exchange of materials between the cortex and the endoplasm (*i.e.* new formation or destruction of cortical materials). As mentioned above, the thickness of the cortex decreases at the polar region and increases at the furrow region during cleavage, whereas the surface area increases at the polar region and decreases at the furrow region (cf. DAN and ONO 1954, HIRAMOTO in press). Although considerable errors are involved in the calculation of the volume of the cortex owing to errors involved in the determination of the thickness, there is no evidence, within the limit of accuracy in the present work, indicating the change in volume in any regions of the cortex during cleavage. MITCHISON (1956) reported no change in thickness of the cortex during cleavage. This result is not in favor of his expanding membrane theory (MITCHISON 1952), because the theory demands the decrease in thickness of the cell membrane in actively expanding regions. The results of the present study are favorable not only for the expanding membrane theory but for other cortical activity theories as well as for the endoplasmic activity theories (cf. HIRAMOTO 1956). Presence of pigment granules in the endoplasm at the furrow region during the late stage of cleavage is an important problem in this connection, but exact nature of the sol-gel change in cytoplasm at this region is not yet investigated in detail.

Summary

1. Methods of determination of the refractive index of the protoplasm in sea urchin eggs were described. The refractive indices of unfertilized and fertilized eggs of *Hemicentrotus pulcherrimus* are of order of 1.40 (cf. Table 1). The cortex has a similar value of the refractive index to that of the endoplasm.

2. The presence of the cortex different in physical properties from the endoplasm was shown by micromanipulation. The thickness of the cortex was determined after the correction of error owing to the refraction of light at the cell surface.

3. The thickness of the cortex is about 3 μ in unfertilized eggs. It increases during development, until it reaches 4 μ shortly before cleavage. It increases at the furrow region and decreases at the polar region during cleavage. After cleavage the difference tends to be compensated.

4. The results were discussed in connection with the thickness of the cortex previously reported and with the problem of new formation of the cell membrane during cleavage.

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